Quantitative Trait Locus Analysis of Growth in *Arabidopsis thaliana*

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Declaration

The following thesis was composed by myself and consists of my own work, which has not been submitted for any other degree or professional qualification.

Abstract

Natural genetic variation found among accessions of *Arabidopsis thaliana* presents the opportunity of locating and identifying novel genes by means of quantitative trait locus (QTL) analysis. In this study, QTL analysis was used to identify loci involved in the genetic control of growth in *A. thaliana*. Non-destructive methods of analysis were developed and used for the measurement of growth rates in roots and leaves, whilst a simple size measurement of mature petals was used to assess growth in the floral organ.

Two putative QTL were identified for primary root length, four for leaf number at day 32 and three for petal size in the Bay-0 x Shahdara recombinant inbred line (RIL) population. The Landsberg *erecta* x Columbia RIL population was also analysed, but no significant QTL were identified.

The analysis suggested that, in all three organs, growth-rate is controlled by multiple small-effect QTL and is a highly plastic trait. Thus, minor environmental fluctuations during the course of experiments can lead to a large environmental variance in measurement of the traits, limiting the power of QTL analyses. Despite minimising these effects by adjusting growth techniques, the numbers and significance of QTL identified in each trait were lower than expected, and for the trait of relative growth rate in leaves no significant QTL were identified.

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Particular thanks goes to my husband, Michael, who, with great patience, has fixed innumerable computing problems, tried to teach me statistics and generally been loving and supportive through all the ups and downs of my postgraduate career. My love to him and to our son, Joel, who has slept beautifully through the writing of this thesis!

Yours, O LORD, is the greatness and the power and the glory and the majesty and the splendour, for everything in heaven and earth is yours... Now, our God, we give you thanks, and praise your glorious name. *1 Chronicles 29:11-13 (NIV).*

List of Abbreviations

AFLP	Amplified Fragment Length Polymorphism
Bay	Bay-0
CAPS	Cleaved Amplified Polymorphic Sequences
Col	Columbia-0
CSS	Chromosome Substitution Strains
Cvi	Cape Verdi Islands
HIF	Heterogeneous Inbred Family
LD	Long Day
Ler	Landsberg erecta
LG	Linkage Group
NIL	Near Inbred Line
QTL	Quantitative Trait Locus/Loci
RFLP	Restriction Fragment Length Polymorphism
RGR	Relative Growth Rate
RIL	Recombinant Inbred Line
SD	Short Day
Sha	Shahdara
SSLP	Simple Sequence Length Polymorphism
STAIRS	STepped Aligned Inbred Recombinant Strain

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1 Introduction

Natural genetic variation can be exploited by means of quantitative trait locus (QTL) analysis to investigate the genetic control of quantitative traits. Self-fertilising plants, such as *A. thaliana*, are particularly suited for study by QTL analysis because of easily produced inbred lines, which allow analysis of homozygous allele effects and the reproduction of genotypes for large sample numbers. The intention of this project was to investigate the genetic control of plant growth by utilising the natural genetic variation found in *A. thaliana*. Growth traits are important fitness-contributors and therefore good candidates for QTL analysis as they are likely targets of selective pressures during adaptation. Adaptation requires the fixation of advantageous alleles, which will vary between populations as selective pressures during adaptation have varied. QTL analysis also offers the possibility of identifying a wide variety of genes which could potentially act in the regulation of plant growth.

1.1 Natural Genetic Variation

A common observation in both plants and animals is that localised populations have adapted to the prevailing climate or environment in which they are situated. This often gives rise to latitudinal clines in traits over a geographical range. For instance, many studies have identified population-based differences in wing length in *Drosophila melanogaster*, which correlate with latitude (van 'T Land, J. *et al.*, 1999). That such adaptations have underlying genetic controls becomes apparent in common-garden experiments, where samples are taken from a variety of geographical locations and grown under a common environment. By such experiments, it was observed that populations of mountain hemlock (*Tsuge mertensiana)* originating from high latitudes in British Columbia had adapted earlier frost-hardiness in comparison to those originating from lower latitudes (Benowicz, A. *et al.*, 2001). Similarly, European Scots Pine (*Pinus sylvestris* L.) populations showed a range of nutrient resorption efficiency during needle senescence, which correlated with latitude. The selection pressure contributing to this adaptation was suggested to be the temperature-related concentration of nutrients available in the soil (Oleksyn, J. *et al.*, 2003).

Genetic variation is often also observed by means of genotyping at neutral marker loci. Fennel pondweed (*Potamogeton pectinatus*) showed latitude-related adaptations that caused variation in plant growth and fitness when plants from different populations were grown in a common environment (Santamaria, L. *et al.*, 2003). These distinct populations (known as 'genets') could be identified by differences in their genetic fingerprints, showing that neutral genetic variation was present between populations (Santamaria, L. *et al.*, 2003). Along with many others, the examples cited above demonstrate that populations frequently adapt to increase fitness in their local environment because of local selective pressures acting on genetic variance.

1.1.1 Natural Genetic Variation in *A. thaliana*

Natural populations of *A. thaliana* are found at a wide range of longitudes and latitudes (Alonso-Blanco, C. & Koornneef, M., 2000; see Figure 1.1). As populations have become established in different locations, they have had to adapt to survive and thrive in the prevailing environmental conditions. These localised, adapted populations are often known as accessions. Many accessions have been collected for use in laboratory studies because they can exhibit interesting variations in phenotypes or carry accession-specific mutations. Over the course of adaptation, alleles arising from advantageous mutations will have become fixed in the genomes of accessions. When accessions are brought from diverse locations into a common environment, the genetic differences which have caused them to adapt to their local environments may become apparent, thereby producing a phenotypic difference between accessions.

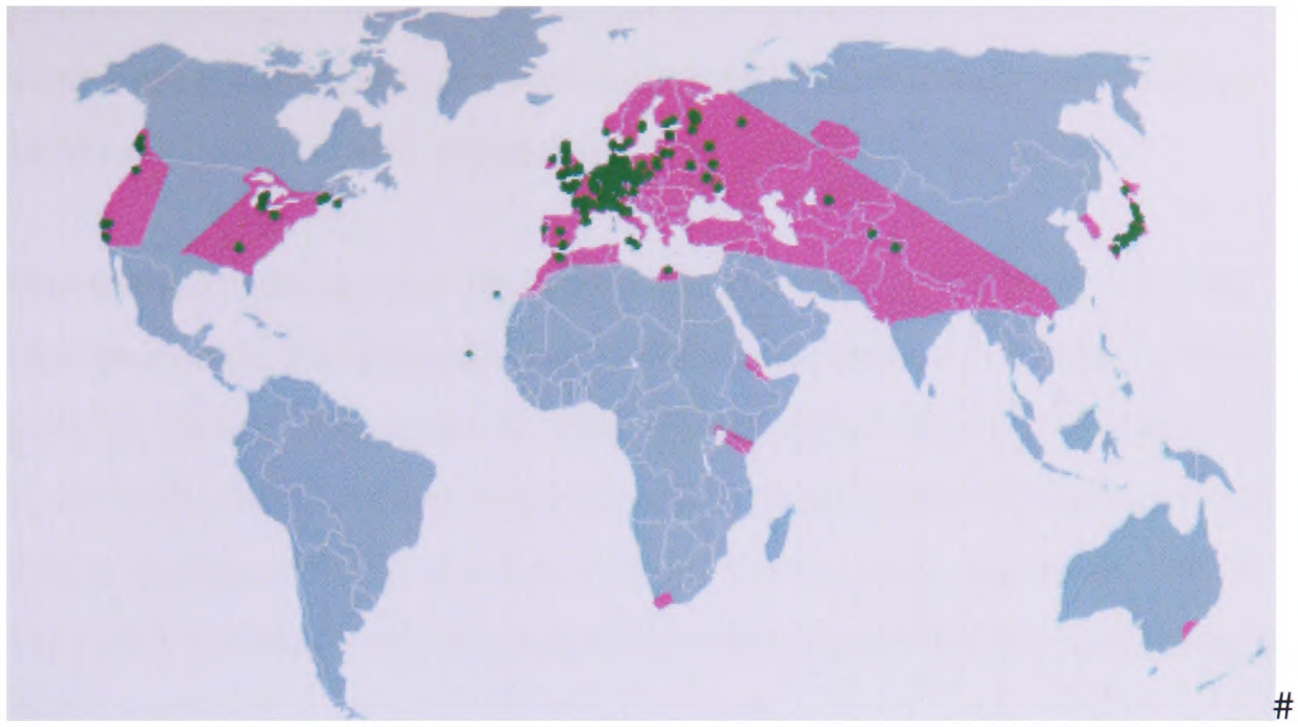


Figure 1.1. The geographical distribution of natural accessions of *A. thaliana*.
Dots show locations from which accessions have been collected for experimental use; shaded areas indicate the general distribution of *A. thaliana* (Alonso-Blanco, C. & Koornneef, M., 2000).

Both phenotypic and underlying genetic differences have been seen in many studies of *A. thaliana* accessions. Two recent studies found natural variation for vernalisation sensitivity amongst accessions of *A. thaliana*, that correlated with latitude (Lempe, J. *et al.*, 2005; Stinchcombe, J. R. *et al.*, 2005). Both involved measuring the dependence of flowering time of natural accessions of *A. thaliana* on the length of the vernalisation period. Accessions were taken from a range of latitudes and brought into a common, controlled environment. Vernalisation sensitivity was negatively correlated with latitude of origin - i.e. accessions from northern latitudes tended to be less sensitive to vernalisation than those originating from nearer to the equator.

Another study of growth-related traits amongst 40 accessions originating from a range of latitudes but grown under a common environment showed significant between-accession variation for cotyledon width, rosette diameter, leaf number, leaf area and dry weight (Li, B. *et al.*, 1998). In general, under the conditions of the experiment, plant size was seen to decrease with increasing latitude, suggesting that accessions had adapted to the latitudinally-dependent local temperatures experienced

at their sites of origin. However, the extent to which latitude could explain the differences observed in plant size was small, suggesting that selective pressures unrelated to latitude had also affected the adaptation of these accessions.

Another study, involving over 100 accessions, also found phenotypic variation amongst accessions, this time referring to the shade-avoidance response (Botto, J. F. & Smith, H., 2002). Phenotypic variation was not correlated with latitude in this study, although a large number of the accessions originated from similar latitudes, potentially limiting this aspect of the analysis. Rather, Botto and Smith (2002) observed wide variation even amongst accessions collected from a single geographic location.

Therefore it appears that both latitude and other local selective pressures can act on the natural variation that arises within *A. thaliana* populations to drive adaptations, which become evident when individuals from different populations are brought into a common environment. This was artificially demonstrated in the laboratory by applying a selective pressure over three generations of *A. thaliana* (Ungerer, M. C. & Rieseberg, L. H., 2003): two inbred lines were crossed to produce a heterozygous F1 generation, which was backcrossed to one of the parental lines. Offspring were subsequently produced by self-fertilisation for three generations under high-density growth conditions. Plants responded to the selective pressure, as observed by a significant difference between the backcross generation and the third selfed generation in viability and fertility at high density. This demonstrated that, if genetic variability is present, local selective pressures can act quickly on *A. thaliana*, producing site-specific adaptations within populations.

Genetic variation between accessions of *A. thaliana* is evident, whether observed through phenotypic effect, as in the studies mentioned above, or by direct genotyping. High within-population genetic variability was observed in Norwegian populations of *A. thaliana* (Stenoien, H. K. *et al.*, 2005). Several maternal families from ten populations ranging from low to high latitude were sampled for genetic variability. Microsatellite variation was found in six of the ten populations: in the most variable of these, half of the loci genotyped were polymorphic and over 70% of

maternal families contained different haplotypes. These findings suggest either that a continuation of gene-flow between populations mixes haplotypes to maintain variability, or, that novel mutations and/or recombination events arise within populations. The former explanation does not appear to be the case in this study, as populations were highly genetically differentiated, even from other populations within 200 metres (Stenoien, H. K. *et al.*, 2005). Sharbel *et al.* (2000) also observed genetic variation between accessions of *A. thaliana* by analysing 79 amplified fragment length polymorphisms (AFLPs) in 142 accessions taken from seven geographical regions. The genetic distance (i.e. the number of genetic differences) between accessions increased significantly with increasing geographical distance (Sharbel, T. F. *et al.*, 2000).

It is likely that, depending on the trait affected there will be latitudinal clines in some areas of variation whilst others show relationships to different environmental or historical aspects of selection. The important aspect for the types of genetic analyses proposed here is the presence of variation between accessions, as has been observed in both neutral and non-neutral parts of the genome.

Analysis of the genetic differences that cause phenotypic differences in a trait amongst accessions can reveal genes that are naturally involved in the control of that trait. This approach has an advantage over traditional reverse genetics approaches as it allows the detection of genes in which knock-out mutations might be unavailable, give no effect or cause a lethal phenotype. It has the potential both to identify novel genes which have not been previously annotated and to assign new functions to known genes. A popular and useful method for the elucidation of genetic control of quantitative traits is quantitative trait loci (QTL) analysis.

1.2 Analysis of Quantitative Trait Loci

The principle behind mapping genes by QTL analysis is that a QTL linked to a marker locus will result in a correlation between the measured phenotype of individuals and their marker genotypes (see Figure 1.2). The population used for QTL mapping must exhibit variation for the trait of interest and genetic variation; a

polymorphic marker linkage map is required for the analysis of neutral genetic variation (Mackay, T. F. C., 2001). Marker loci should be polymorphic, evenly spread and give good coverage of the genome. Easily genotyped loci, such as simple sequence length polymorphisms (SSLPs) are preferable, because much genotyping is involved in the preparation of lines for mapping. The accuracy of QTL mapping is dependent on the extent of marker loci coverage and the number of recombination events which have taken place in the population. Few recombination events will fail to disrupt distant marker-QTL associations and so give a very broad confidence interval for any QTL location. Similarly a low density of markers will reduce the ability to detect QTL as multiple recombination events might occur between markers. These events cannot be detected but, if they occur, will cause disruption of the QTL-marker association, therefore their probability should be minimised.

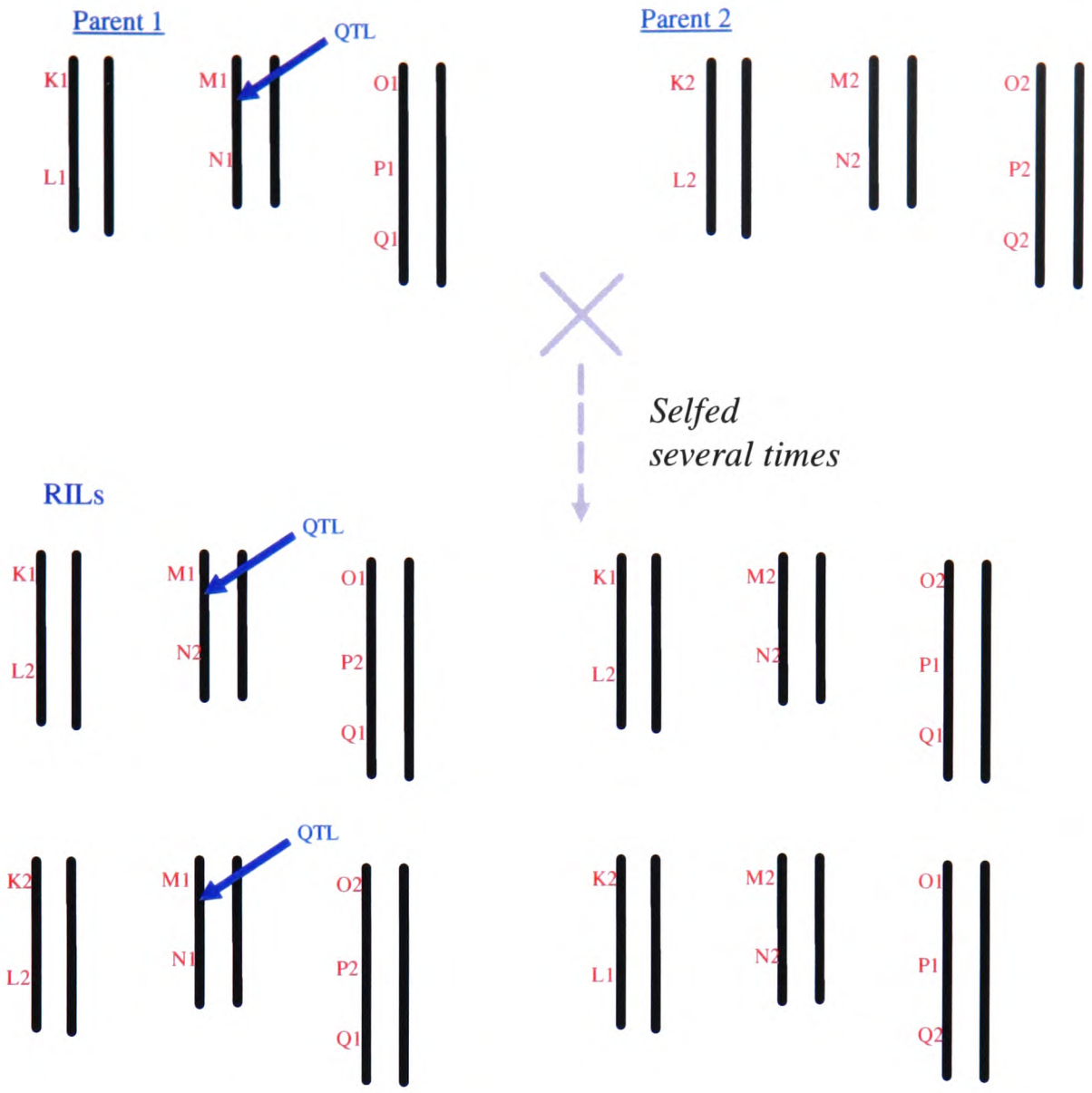


Figure 1.2. The principle of QTL mapping using recombinant inbred lines (RILs).
Markers K to Q, shown in red, are known by genotyping to be either from parent 1 or parent 2. All loci in parents and RILs are homozygous. Examples of the homozygous offspring developed from this cross for mapping are shown. In the offspring, the QTL will be randomly distributed with most of the markers, but linkage would give non-random association with marker M1. Hence, when the trait to which the QTL contributes is measured, a tendency in this trait (e.g. faster growth rate) will show non-random association with allele M1.

1.3 Recombinant Inbred Line Populations

Predominantly selfing plants, such as *A. thaliana*, provide a suitable tool for QTL analysis because of the possibility of producing a recombinant inbred line (RIL) population (Alonso-Blanco, C. & Koornneef, M., 2000). A RIL population is produced by crossing two genetically different parent plants, then repeatedly selfing individuals from the F1 generation. Heterozygosity is gradually lost with each

generation of selfing, resulting in 99.9% homozygous plants by the 10th generation (Falconer, D. S. & Mackay, T. F. C., 1996). RILs are permanent resources, because they are virtually homozygous throughout their genome; continued selfing of a RIL does not result in any change in genotype. Therefore a RIL population can be genotyped once and used for repeated analyses.

In *A. thaliana*, various RIL populations have been developed and used for QTL analyses in a diversity of traits. For example, a RIL population produced from the parental accessions Landsberg *erecta* (L*er*) and Columbia (Col-0) (Lister, C. & Dean, C., 1993) has been used to map QTL for various traits, including floral morphology (Juenger, T. *et al.*, 2000), circadian system control (Swarup, K. *et al.*, 1999), sodium chloride tolerance (Quesada, V. *et al.*, 2002) and resistance to rabbit herbivory (Weinig, C. *et al.*, 2003). The Cape Verde Islands (Cvi) x L*er* RIL population (Alonso-Blanco, C. *et al.*, 1998) has also been extensively used to analyse traits including life-history traits such as seed size (Alonso-Blanco, C. *et al.*, 1999), light and hormone responses (Borevitz, J. O. *et al.*, 2002) and phosphoglucomutase activity (Sergeeva, L. I. *et al.*, 2004). Circadian system control has been studied in both the L*er* x Col and Cvi x *Ler* RIL populations (Swarup, K. *et al.*, 1999) and trichome density (Symonds, V. V. *et al.*, 2005) has been analysed in both these and the Bay-0 x Shahdara RIL population (Loudet, O. *et al.*, 2002). Analysis of traits across different RIL populations and in different environments is important for a complete detection of QTL, because important loci will go undetected if there is no variation between the parents of the RIL population under analysis, and QTL-environment interactions can cause QTL effects to appear in some conditions but not in others. However, the diversity of traits analysed by this method illustrates the wide potential of QTL analysis in RIL populations.

The RIL populations used in this study are the Bay-0 x Shahdara (Bay x Sha) and Landsberg *erecta* x Columbia (L*er* x Col) RIL populations.

1.3.1 The Bay-0 x Shahdara RIL Population

The Bay x Sha RIL population was produced by Olivier Loudet and Sylvain Chaillou at the INRA (*Institut National de la Recherche Agronomique*) in Versailles (Loudet, O. *et al.*, 2002). Bay-0 and Shahdara parent plants were crossed to produce a heterozygous F1 population. One F1 plant was self-fertilised. From the F2 generation, multiple lines were taken through single seed descent until the F6 generation. Homozygosity would then be estimated at 0.984, according to the equation,

$$\mathbf{F_t = \tfrac{1}{2}\,(1 + F_{t-1})} \qquad \textit{Equation 1}$$

$\mathbf{F_t}$ = the homozygosity at generation **t**;
$\mathbf{F_{t-1}}$ = the homozygosity at generation **t-1**
(Falconer, D. S. & Mackay, T. F. C., 1996).

F6 seeds were genotyped at 38 SSLP marker loci (see Figure 1.3) to produce a RIL database and then selfed once more to produce F7 seeds for each individual RIL (Loudet, O. *et al.*, 2002). The Bay-0 and Shahdara accessions were taken from Germany and Tajikistan, respectively; latitudinal, ecological and genetic diversity were apparent in this cross (Loudet, O. *et al.*, 2002). The F1 seeds should therefore exhibit high heterogeneity, and a good degree of diversity should be present in the resulting RILs, making this a good population for QTL analysis.

QTL analysis using the Bay x Sha RIL population has already been published for flowering time (Loudet, O. *et al.*, 2002), nitrogen metabolism associated growth traits (Loudet, O. *et al.*, 2003b) and water and anion contents in leaves in response to nitrogen availability (Loudet, O. *et al.*, 2003a). Also, various traits are currently being studied, including seed-germination, aluminium tolerance, trichome density and powdery mildew resistance (see www.dbsgap.versailles.inra.fr/vnat/Documentation/33/DOC.html). The success of these previous analyses and the number and diversity of traits now being studied in this RIL population are indicative of the presence and usefulness of genetic and phenotypic diversity. QTL analyses of root architecture are also currently underway (Loudet, O. *et al.*, 2005) and are discussed in Chapter 4.

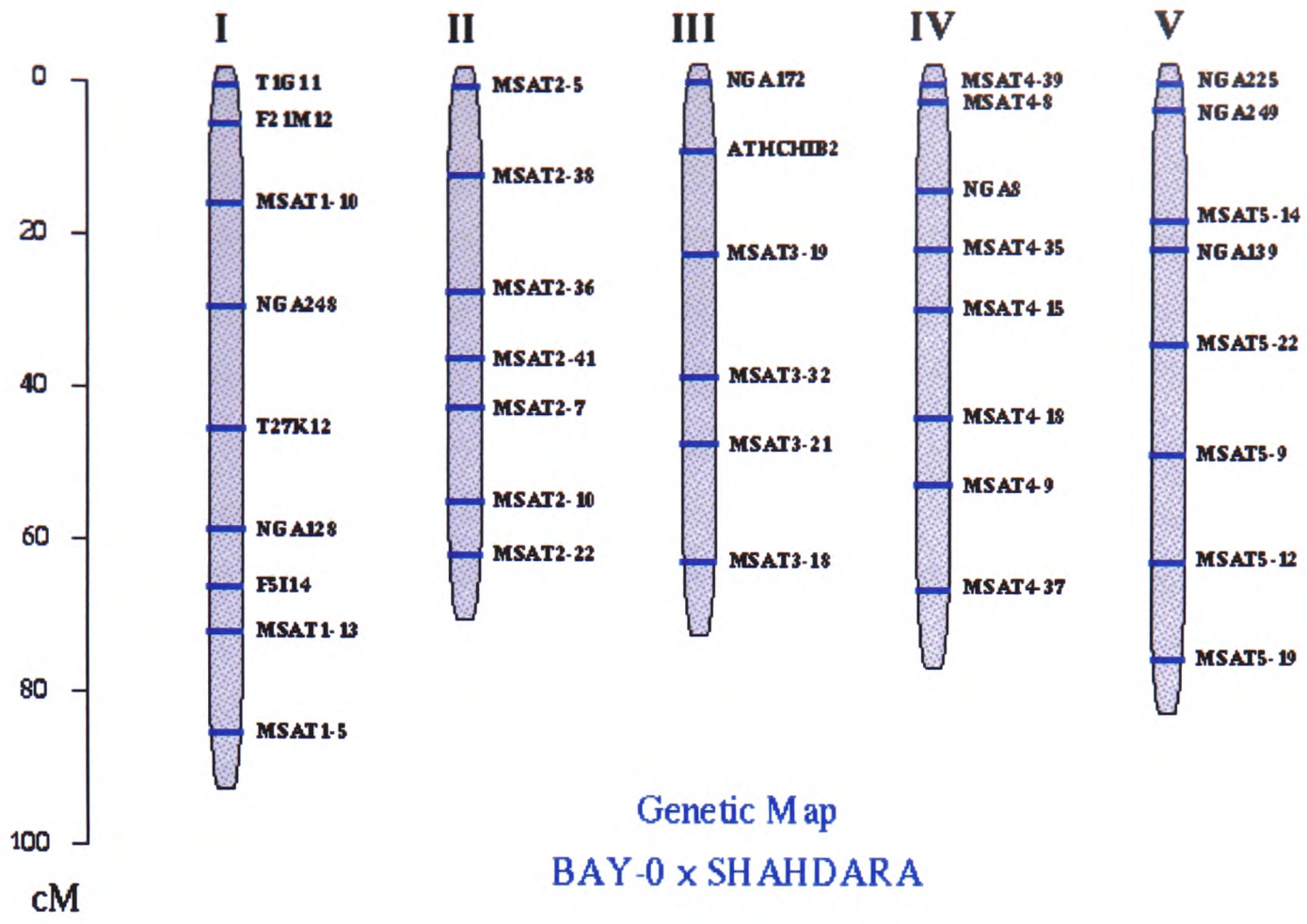


Figure 1.3. Genetic map showing markers used in the Bay-0 x Shahdara RIL population.
(Loudet, O. *et al.*, 2005)

1.3.2 The Landsberg *erecta* x Columbia RIL population

By a similar approach, the L*er* x Col RIL population was produced by Lister and Dean (1993) and has since been used in the analysis of many quantitative traits, some of which were mentioned above. Landsberg *erecta* and Columbia are two very commonly used accessions of *A. thaliana* that differ for many traits, including height, rosette size, leaf shape and flowering time (Lister, C. & Dean, C., 1993). Originally 64 restriction fragment length polymorphisms (RFLPs) were mapped and genotyped in 100 RILs of the L*er* x Col population, but many more markers have since been added to create a high density marker map (Lister, C. & Dean, C., 1993). Availability of the sequence of the Columbia and Landsberg *erecta* genomes (http://www.arabidopsis.org.uk) also accommodates the identification of new polymorphic markers for genetic mapping in the L*er* x Col RIL population and aids

in the identification of candidate genes within QTL confidence intervals by the presence of non-synonymous substitutions. One possible drawback of using this RIL population for QTL analysis is the presence of the *erecta* mutation, which causes an obvious morphological difference between the parental strains and which could mask smaller-effect QTL in the traits to which *ERECTA* contributes. This was probably the case in an analysis that recorded a single QTL for height at flowering in the *A. thaliana* L*er* x Col RIL population: the QTL mapped to the *ERECTA* locus and explained most, but not all of the variance in height, suggesting that other loci also contributed to height variation but were not detected because of the small magnitudes of their effects in comparison to the large effect of the *ERECTA* locus (Kearsey, M. J. *et al.*, 2003).

1.4 The extent and limitations of QTL Analyses

Few QTL studies have been extended to the point of identifying individual genes. The most likely method for identification of the gene responsible for a QTL is by analysis of candidates within a QTL confidence interval after fine-mapping has sufficiently delimited this interval. One such example is the identification of a gene controlling heading date in rice. A region on the short arm of chromosome 6 was originally identified in a QTL mapping experiment using a cross between the rice cultivars Nipponbare and Kasalath (Yano, M. *et al.*, 1997). NIL analysis resulted in the segregation of two QTL within this region – Hd3a and Hd3b. The extent of the Hd3a region was further reduced by fine-mapping with cleaved amplified polymorphic sequence (CAPS) markers, which produced a confidence interval of about 20kb containing the Hd3a QTL (Kojima, S. *et al.*, 2002). Of the four genes predicted within this region, one gene (*CDS25*) was selected as a likely candidate because of its high similarity to the *Flowering Time* (*FT*) gene, which promotes flowering in *A. thaliana* under long day (LD) conditions. Further investigations confirmed that this was the gene responsible for the Hd3a QTL: RT-PCR showed expected expression patterns for a promoter of heading in short day (SD) conditions; non-synonymous sequence variation was identified between alleles of the parent cultivars; complementation experiments showed that introduction of the Kasalath transgene caused earlier heading than introduction of the Nipponbare transgene in

SD conditions; and over-expression of *Hd3a* from the 35S enhancer promoted early heading in transgenic plants.

Hd3a is an example of a relatively small effect QTL that has been identified – the Nipponbare allele of the *Hd3a* QTL was originally estimated to reduce heading date by 2.1 days compared to a population mean of 125.7 days to heading (Yano, M. *et al.*, 1997). Mapping this QTL to a gene was facilitated by fine-mapping, which significantly delimited the QTL confidence interval, whilst the low density of genes within this fine-mapped region allowed quick identification of the gene responsible.

Another example of a QTL successfully identified to nucleotide substitution level is that of the photoperiod receptor *CRY2*. A QTL that caused early flowering in SD conditions had been provisionally mapped to the top of chromosome 1 in *A. thaliana* using the Cvi x L*er* RIL population. This QTL was fine mapped to a 45kb region, which contained fifteen open reading frames, including *CRY2*, a gene known for its function in control of flowering time and perception of day length (El-Assal, S. E.-D. *et al.*, 2001). Through complementation experiments and sequencing this gene was shown to be responsible for the QTL effect, which was caused by a single nucleotide substitution in the Cvi accession of *A. thaliana*. Thus a new allele of the *CRY2* gene was identified through QTL analysis, but the function of the CRY2 protein had been previously annotated.

Examples of such 'complete' QTL analyses are rare, however. More often, analyses arrive at a general description of genetic architecture with wide confidence intervals for the positions of QTL. The next stage of analysis – to reduce the confidence interval and identify and test candidate genes – can be extremely laborious and time consuming. For example, in 2002, data was published from a study of leaf traits using a population of 100 RILs (Perez-Perez, J. M. *et al.*, 2002). Twenty-one QTL were identified – some involved in juvenile leaf traits, some in adult leaf traits, whilst eight affected traits in both juvenile and adult leaves. The amount of phenotypic variation explained by a QTL varied from 2.3% to 44.1% with QTL confidence intervals ranging from 4.4cM up to 44.4cM. For some of the major effect QTL the authors were able to predict candidate loci, based on genes known to be involved in

leaf morphology traits that mapped within the QTL intervals. For example, *ERECTA* is likely responsible for the QTL effect observed between 48cM and 58cM on chromosome 2 as this effect correlated with the *ERECTA* locus, which was used as a marker during the analysis. For the novel QTL, however, no further identification has yet been achieved and for those that are only responsible for a small proportion of the observed variance in a trait, it seems unlikely that any further identification will take place in the near future.

This limitation is observed frequently in QTL analyses as many loci are mapped to wide confidence intervals, but few studies extend to the ultimate aim of QTL analysis – the actual identification of the gene and locus variation responsible for the observed effect.

1.4.1 QTL for Growth-related Traits

Plant growth rate is a highly important trait in agriculture. Traditionally, fast-growing varieties of crops have been selected for in order to improve yield. In one study of wheat, QTL analysis has been used to identify loci under selection during ancient development of agriculture by mapping QTL for 'domestication' traits, such as loss of brittle rachis (the tendency of seed pods to break and shed seeds at maturity), increased seed size, altered developmental timings and increased yield (Peng, J. H. *et al.*, 2003). The genetic variation between a domesticated cultivar, *Triticum durum* and a wild progenitor, *T. dicoccoides* was investigated. Several QTL were mapped for most of the traits, and seven regions were identified to which clusters of QTL for various traits were mapped, suggesting either tight linkage of QTL or pleiotropy (one QTL explaining variation in several traits). Clustering of QTL was also found in the genetic analysis of sunflower domestication (Burke, J. M. *et al.*, 2002), where it became apparent that negative-effect alleles had been carried by linkage during selection for positive-effect alleles during domestication. QTL analyses bring about the possibility of identifying and separating such antagonistic effects of physically linked QTL to improve the precision of selection in agriculture.

Many genes for growth-related traits have been identified through the study of developmental mutations. However, QTL analyses will potentially enable the identification of genes that remain hidden through genetic redundancy or cause lethal phenotypes in knock-out studies. Also, QTL analyses will identify natural variants of genes, rather than induced mutations, and can identify novel alleles of previously annotated genes, as illustrated by the example of the *CRY2* allele, above (El-Assal, S. E.-D. *et al.*, 2001).

A novel gene for root growth was identified by QTL analysis in *A. thaliana* (Mouchel, C. F. *et al.*, 2004). The effect of a natural mutation in *BREVIS RADIX* (*BRX*) was first observed in the short root phenotype of Umkirch-1, and confirmed to be caused by a single recessive locus by the 3:1 segregation of phenotypes observed in the F2 generation of an Umkirch-1 / Slavice-0 cross. *BRX* was mapped by QTL analysis and use of a Near Isogenic Line (NIL) introgression. It was confirmed by candidate gene analysis to correspond to At1g31880, a member of a previously un-described plant-specific gene family and a potential transcription factor (Mouchel, C. F. *et al.*, 2004).

The effect of the Umkirch-1 allele of *BRX* is likely due to a single mutation resulting in a premature stop codon: this single locus explained about 80% of the total phenotypic variation in root length observed across the Umkirch-1 x Slavice-0 RIL population. Such large-effect mutations rarely become fixed in populations because of their potential reductions in fitness.

Similarly, a novel gene for the control of tomato fruit weight was identified by QTL analysis: *fw2.2* was mapped to a region of chromosome 2 in two populations formed from crosses between a domesticated variety of tomato and a wild relative. Between 30% and 47% of the total phenotypic variance for fruit weight in these populations was explained by the QTL, indicating a large-effect QTL (Alpert, K. B. *et al.*, 1995). Using NILs segregating for the domesticated and wild alleles of this gene, the activity of *FW2.2* has been analysed, showing that the domesticated allele causes increased fruit size whilst reducing the total number of fruit produced, but that its

effect on fruit size is one of direct control, rather than an indirect consequence of the reduced number of fruit (Nesbitt, T. C. & Tanksley, S. D., 2001).

BREVIS RADIX and *FW2.2* confer large additive effects, which greatly simplifies the task of fine mapping and identification of QTL. However, the sizes of QTL effects can vary considerably and are more often small. Although many QTL are found in initial analyses, only the few that exhibit the largest effect on variation tend to be further identified and characterised. For example, *FW2.2* was one of 11 QTL identified in the initial mapping analysis (Paterson, A. H. *et al.*, 1991), but appears to be the only one that has been fine-mapped and characterised (Alpert, K. B. & Tanksley, S. D., 1996).

Of course, it is easier to identify genes that exhibit a large quantitative effect or even a qualitative effect as they will be phenotypically obvious, even with small sample numbers. Such genes can often be identified by means of reverse genetic methods, such as mutant screens. For a highly plastic trait such as growth rate, however, which must respond effectively to minor fluctuations in the plant's environment, models suggest the contribution of multiple small-effect genes (Remington, D. L. & Purugganan, M. D., 2003). Dissecting such traits will require large sample numbers for statistical significance, a very constant environment in order to reduce any environmental variance acting on the plasticity of the trait and a thorough QTL analysis in order to locate the multiple small-effect QTL responsible for genetic variation.

1.5 Candidate Genes for Growth Rate Regulation

Ultimately, plant growth occurs when cells divide and expand; organ growth rate is dependent on the rates at which these processes happen. In plants, cell division occurs in meristems – zones of self-perpetuating stem cells that supply cells for the development of new organs (den Boer, B. G. W. & Murray, J. A. H., 2000). The rate of tissue production depends on the extent of meristematic activity (i.e. the number of cells capable of division and expansion), as well as the rate of cell division and rate and extent of cell expansion. Furthermore, organ growth is regulated by external

signals such as nutrient availability and plant hormones, coordinating organ growth with the whole-plant environment (Beemster, G. T. S. *et al.*, 2003).

1.5.1 Cell division

Cell division occurs upon completion of the mitotic cell cycle. Cell cycle progression is controlled by the cyclic activity patterns of cyclin-dependent kinases (CDKs), which are dependent on cyclins, CDK-activating kinases (CAKs) and CDK Inhibitors (CKIs; Potuschak, T. & Doerner, P., 2001). Generally it appears that *CDK* gene activity itself does not appear to modify cell division activity (den Boer, B. G. W. & Murray, J. A. H., 2000), although a positive correlation between CDKA protein concentration and root growth rate was found in a few fast growing accessions of *A. thaliana* (Beemster, G. T. S. *et al.*, 2002). More likely, cyclins and other CDK regulators will be important modifiers of plant growth through their regulation of CDKs (den Boer, B. G. W. & Murray, J. A. H., 2000; Potuschak, T. & Doerner, P., 2001).

For example, Cyclin D2 in *A. thaliana* has been found to increase growth rate by shortening the G1 phase of the cell cycle when expressed in tobacco plants (Cockcroft, C. E. *et al.*, 2000). Also, D-type cyclins have been found to regulate the cell cycle in response to extra-cellular signals such as plant hormones and nutrient availability (den Boer, B. G. W. & Murray, J. A. H., 2000), thus linking cell division regulation to the external environment.

1.5.2 Meristematic competence

Additionally, growth rates may be regulated by modulation of meristem size, such as by *ARGOS*, which regulates organ size by temporally regulating the meristematic competence of cells (Hu, Y. X. *et al.*, 2003). Enhanced expression of *ARGOS* sustains cell division in leaf primordia (i.e. it increases the duration of meristematic competence of cells), leading to larger organs with an increased cell number. *ARGOS* is also involved in response to extra-cellular signals, as its expression is induced in response to the plant hormone auxin.

1.5.3 Cell Expansion

The duration and extent of cell expansion will affect the final size of organs, while the rate of cell expansion will affect organ growth rate. In roots, cell expansion begins in the basal region of the meristem, during the last round of cell division; thereafter, cells expand rapidly in the elongation zone (Beemster, G. T. S. *et al.*, 2003). As indeterminate organs, roots continue to grow throughout their life-span in an essentially linear manner. Leaves, on the other hand are determinate organs, produced from leaf primordia, which form on the surface of the shoot apical meristem. In leaves, cell expansion is not linear; rather cells expand both longitudinally and laterally in a controlled manner. Two genes involved in the control of leaf cell expansion were identified through mutant analysis as *ANGUSTIFOLIA* and *ROTUNDIFOLIA*, which control cell expansion independently in the leaf width and leaf length direction respectively (Tsuge, T. *et al.*, 1996). One model has shown that under constant conditions, final leaf area correlates with cell number, which is established by cell division during the first two-thirds of leaf development (Cookson, S. J. *et al.*, 2005), suggesting that cell expansion does not regulate leaf growth. However, cell expansion, which is responsible for the remainder of leaf expansion, is likely to be highly plastic, thus linking leaf growth to the external environment of the plant and being an important factor in the control of leaf growth in natural, variable environments.

1.5.4 Polyploidy

One mechanism by which cells can increase in size is endopolyploidy, whereby cells undergo rounds of chromosomal duplication without mitosis, known as endoreduplication. Nuclear volume increases in direct proportion to ploidy (the number of copies of DNA in the nucleus), but cellular volume can experience a much greater increase than the respective increase in ploidy (Sugimoto-Shirasu, K. & Roberts, K., 2003). In a study of stem and leaf epidermal pavement cells, it was found that 71% of cells underwent endoreduplication, and this was always associated with an increased cell size (Melaragno, J. E. *et al.*, 1993). However, Beemster *et al.* (2002) found no correlation of cell size with ploidy in the roots of 18 accessions of *A. thaliana*. Also, over-expression of a dominant negative mutation of *CDKA* reduces

endoreduplication in maize endosperm but has no effect on cell size or final organ size (Leiva-Neto, J. T. *et al.*, 2004). So it remains uncertain whether modifying endoreduplication will affect plant growth rates.

1.5.5 Extra-cellular Signalling

A common theme in regulation of the above mechanisms of growth is their links to the extra-cellular environment. As sessile organisms, plants must be able to sense their local environment and respond accordingly. Plant hormones are intrinsic to the control of organ growth and may be involved in coordinating the growth of organs with the rest of the plant and its environment. For example, auxin promotes leaf initiation from the SAM (Reinhardt, D. *et al.*, 2000) and induces lateral root formation (Lopez-Bucio, J. *et al.*, 2003); ethylene inhibits growth of the root and hypocotyl in dark-germinated seedlings; other signalling molecules, including giberellic acid and abscisic acid, play important roles in the development of the plant (Chow, B. & McCourt, P., 2004). Also environmental conditions such as nutrient availability, light intensity, day length and temperature can affect the growth of plants. In root growth, for example, directional growth of lateral roots is regulated in response to the availabilities of nitrate and phosphate in the soil (Lopez-Bucio, J. *et al.*, 2003). The ability of a plant to modulate its growth – in terms of both rate and direction – in response to its environment is critical for its survival and fitness.

Such extra-cellular regulatory factors might be particularly significant in this QTL study as it utilises natural accessions that have adapted to different environments. Changes in the production of and response to these signalling molecules would be an effective way for a plant to adapt to its local environment. QTL for variation in environmental-responses are likely to become apparent when a RIL population is grown in a uniform environment.

1.5.6 Common versus specific controls of organ growth

An advantage of investigating growth rate in multiple organs of the plant is that it introduces the potential of distinguishing between common and specific controls of organ growth. Co-localisation of QTL for growth rate in different organs would

suggest a gene that is involved in the intrinsic control of growth rate – factors such as cell cycle regulation. Organ specific QTL are more likely to be involved in response of that organ to the environment, because leaves, roots and petals are in different environments, which will promote the responses of different QTL. However, QTL that co-localise may not be produced by a single gene: distinguishing between pleiotropy and clustering of QTL will require fine-mapping with many recombinant lines.

1.5.7 Summary of candidates for QTL analysis of growth rate

Therefore cell-cycle genes, such as D-type cyclins, and their corresponding upstream regulators and signalling molecules are likely to be imperative in growth rate control as well as genes that control meristem size and competency. These, along with genes involved in more general aspects of environment-sensing and signalling, are possible candidates for identification in this QTL analysis. The investigation of growth rate in three organs of *A. thaliana* may allow the comparison of intrinsic and organ-dependent growth controls, but this analysis will be limited by the RIL population and environmental conditions utilised.

1.6 Plasticity

Quantitative traits, such as growth rate, in plants tend to be highly plastic – that is they are able to adjust in response to the local environment of the plant. Whilst this might increase the scope of QTL analysis in detection of QTL that respond to certain environmental cues, it can also be a limiting factor in QTL mapping. *A. thaliana* has been observed to respond to many different environmental factors, all of which require regulation in a controlled-environment experiment.

Examples of the plasticity inherent in the growth and development of *A. thaliana* include sensitivity to nutrient concentrations, air quality, temperature, water and light. The root system of the plant is particularly responsive to its local environment, in order to enhance fitness and to best compete in the highly variable conditions that exist in soil. For example, roots respond to local nitrogen levels by producing lateral roots towards hotspots of nitrogen, whilst low phosphate conditions stimulate the

growth of root hairs, which give plants a competitive advantage in low phosphate (Lopez-Bucio, J. *et al.*, 2003). Leaf morphologies and flowering time can show sensitivity to ambient carbon dioxide levels (Zhang, J. & Lechowicz, M. J., 1995), temperature (Hoffmann, M. H. *et al.*, 2005) and vernalisation treatments (Lempe, J. *et al.*, 2005; Stinchcombe, J. R. *et al.*, 2005). Intuitively, water and light availability will affect plant growth: the ability of root systems to adapt in response to osmotic stress might give them a competitive advantage in drought conditions (Gerald, J. N. F. *et al.*, 2006), whilst young seedlings especially need to adjust development in response to light quantity and quality in order to compete successfully with surrounding plants (Dorn, L. A. *et al.*, 2000; Sessa, G. *et al.*, 2005; Smith, H. & Whitelam, G. C., 1997).

So the plant can respond to diverse aspects of its environment, and its fitness and competitive ability may depend on its sensitivity to some of these external signals. This sensitivity of response (or degree of plasticity) can vary, however, between genotypes. An assessment of the fitness of 33 accessions of *A. thaliana* in limiting carbon dioxide conditions showed natural variation amongst these accession in their ability to adapt, measured by length of survival (Sharma, R. K. *et al.*, 1979). The ability of the root system to respond to osmotic stress was also investigated, showing that Columbia plants were more sensitive to osmotic stress than Landsberg *erecta* genotypes (Gerald, J. N. F. *et al.*, 2006). A study of several morphological characters in 74 accessions showed that general responses of *A. thaliana* to temperature changes – faster growth, fewer, smaller leaves, more leaf hairs and longer sepals at 22°C compared to 14°C – also showed a large degree of variation across accessions (Hoffmann, M. H. *et al.*, 2005).

Plasticity may have two significant effects. Firstly, the ability of plants to adapt to their local environment may mask some of the intrinsic controls of quantitative traits, as the variation expected between genotypes when brought into a common environment will not be observed if they are highly plastic, as all plants would adapt to best growth and fitness in their new environment, thus appearing phenotypically identical. Secondly, small fluctuations in the environment may produce phenotypic differences due to environmental response, rather than genetic differences in trait

control, thus complicating the expected phenotype-genotype correlation of QTL analysis. In order to reduce this limitation, the environment must be kept as constant as possible during an analysis, several samples of each genotype should be used and plant positions should be randomised and mixed regularly during the experiment in order to avoid environmental bias produced by uncontrollable differences of local environment across the experimental area.

1.7 Aim

The aim of this study was to investigate the genetic control of growth rate in *A. thaliana*. For this, QTL analyses were undertaken of growth rate in roots and rosettes and of size in petals using two RIL populations and a set of STepped Aligned Inbred Recombinant Strains (STAIRS). During the course of the experiments adjustments have been made to experimental techniques in order to reduce environmental variance and thereby increase the power of the QTL analyses. The following chapters describe and discuss the results of these experiments.

2 Materials and Methods

2.1 Seed sterilisation

In all cases, before sowing, seeds were sterilised in 70% EtOH with Tween20 (1 µl Tween20 per 250 ml 70% EtOH) for 15 minutes then washed twice with 90% EtOH. Seeds were spread on sterile filter paper to dry completely before being either spread on solidified agar or suspended in 0.1% agarose.

2.2 Seed planting / plant growth conditions

After sterilisation seeds were stratified for 72 hours at 4° C, to encourage uniform germination, then moved to growth positions for germination.

2.2.1 Root measurements

For root growth measurements, seeds were suspended in 0.1% agarose and then sown onto square 1% agar plates containing 0.5 × Murashige & Skoog basal salt mixture (Murashige, T. & Skoog, F., 1962) and 0.6% (w/v) sucrose. In early experiments, *A. thaliana* salts (ATS medium) and 0.5 × Johnson medium were also used for root growth measurements (see Table 2.1). Seeds were equally spaced along a horizontal line about 25 mm from the top of the plate (see Figure 2.1). The 0.1% agarose suspension allowed the precise positioning of seeds using a 100 µl Gilson pipette. An equally sized drop of suspension around each seed was aimed for. For QTL analysis 12 seeds of each genotype were sown onto two plates, six on each plate; in HIF analyses, pairs or groups of genotypes were alternated across each plate. Plates were positioned in a random order in the growth area. These measures were to take into account any potential influence of individual plates or positions on the growth measurements. Plates were positioned vertically in a long-day (LD; 16 hour light, 8 hour dark) culture room for germination.

2.2.2 Rosette and petal measurements

For rosette or petal measurements, seeds were germinated on flat Petri dishes of autoclaved 0.6% agar in distilled water (dH_2O). After stratification plates were

transferred to a growth room under either short-day or long-day conditions, depending on the nature of the experiment. One week after transfer, seedlings were transplanted to soil. Plants remained under the same growth conditions for the entirety of their vegetative growth phase. Up to ten individuals of each genotype were grown in a randomised block design and plants were randomly shuffled at intervals through the growth phase so as to reduce any local environmental effects.

Compound	ATS concentration (μM)	0.5 × Johnson concentration (μM)
KNO_3	5000	3000
KH_2PO_4 (pH5.5)	2500	
$MgSO_4$	2000	
$Ca(NO_3)_2.4H_2O$	2000	2000
$NH_4H_2PO_4$		1000
$MgSO_4.7H_2O$		500
Fe-EDTA	50	
KCl		25
H_3BO_3	70	12.5
$MnSO_4.H_2O$		2.5
$FeSO_4.7H_2O$		2.0
$MnCl_2$	14	
$CuSO_4.5H_2O$	0.5	0.25
$ZnSO_4.7H_2O$	1.0	1.0
H_2MoO_4		0.05
Na_2MoO_4	0.2	
NaCl	10	
CoCl2	0.01	

Table 2.1. Composition of media used during seed germination and early growth.

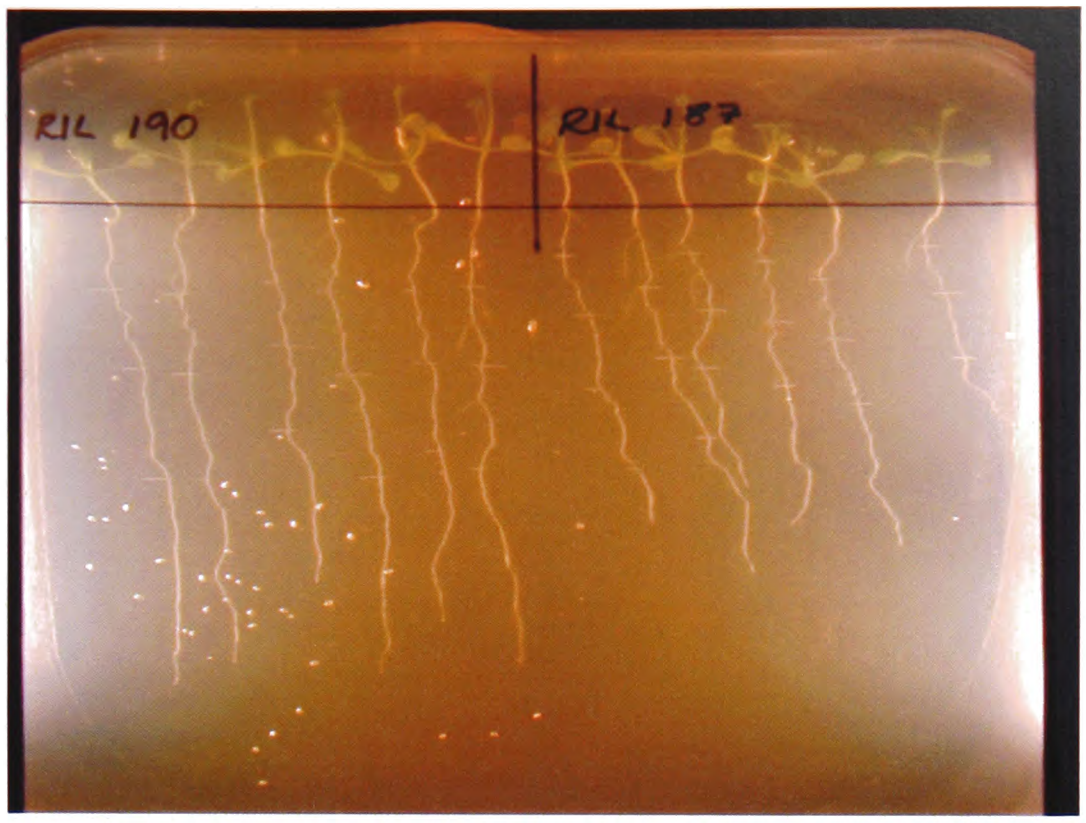


Figure 2.1. Example of two Bay x Sha RIL genotypes growing on a vertical agar plate for root measurements.

2.3 Measurements

Root lengths were measured by scoring the position of the root tip on the back of the plate with a razor blade from the 3rd until the 12th day after transfer of plates to the culture room. Plates were then photographed and roots measured using *Image Tool* (Wilcox, D. *et al.*, 1995).

Rosette areas were measured by aerial photography. The photos were analysed using *Photoshop* and *Image Tool*: photographs were first transformed into black and white images by selecting the green rosette areas using the magic wand tool in *Photoshop 3.0* (Adobe Systems Incorporated, 2004; see Figure 2.2); rosette areas were then measured on the transformed images by using the 'Find Object' command in *Image Tool* (Wilcox, D. *et al.*, 1995), thresholding the image and selecting the white rosette area. A green cardboard marker of known area was included in each photograph and treated as rosettes for correlation of the pixel number to actual area.

Petals were dissected from the 4th to 12th flowers of the main inflorescence. Dissection was done in the morning, selecting flowers whose petals were fully reflexed, so that all petals were measured at the same stage of growth. Petals were

laid on a thin layer of 1% agar on a microscope slide. Images were taken under a microscope and analysed in *Photoshop 3.0* and *Image Tool* in the same way as rosette photographs.

Figure 2.2. Rosette images.
(Above) aerial photograph of *A. thaliana* growing for rosette area measurements and (below) photograph transformed to black and white for measurement.

2.4 QTL analysis

QTL analyses were carried out in *QTL Express*, a web-based programme developed at the University of Edinburgh (http://qtl.cap.ed.ac.uk; Seaton, G. *et al.*, 2002). As a mapping programme specific to the use of RILs has not yet been developed, F2 Inbred analysis was used as the best available approximation to a RIL population.

2.5 Statistics

Statistical analyses – analyses of variance (ANOVA), Student's *t*-tests, Pearsons correlations and calculations of means, variances and standard deviations – were performed in *Minitab 14* (Minitab Inc., 2003) and in *Microsoft Excel* (Microsoft Corporation, 2002).

2.6 Genotyping

2.6.1 DNA Extraction

For DNA extraction, two or three young leaves were selected from each plant. Fresh leaf material was ground in a 1.5 ml microcentrifuge tube at room temperature, 400 µl of extraction buffer (200 mM Tris HCl pH 7, 250 mM NaCl, 25 mM EDTA, 0.5% SDS) was added and the tube was vortexed for 5 seconds. Samples were left at room temperature for up to or over one hour, then spun at 13,000 rpm for 1 minute. 300 µl of the supernatant was collected and added to 300 µl of Isopropanol. This was gently mixed and left at room temperature for 2 minutes, then spun again at 13,000 rpm for 5 minutes to pellet the DNA. Supernatant was removed, samples were washed in 70% EtOH and dried in a sterile hood. DNA was resuspended in 100 µl of sterile water with 0.1 µl of RNase and stored at -20° C.

2.6.2 PCR

The following PCR conditions were used for amplifications:

Average PCR mix:	1 µl PCR buffer (10x)
	0.2 µl dNTPs

	0.2 µl forward primer	
	0.2 µl reverse primer	
	0.01 µl Taq	
	1 µl DNA	
	7.39 µl dH_2O	
Average PCR programme:	2 minutes at 94°C	*Initial denaturing step*
	15 seconds at 94°C	*Denaturing step*
	30 seconds at 50°C	*Annealing step*
	45 seconds at 72°C	*Elongation step*
	repeat steps 2 – 4 thirty times	
	2 minutes at 72°C	*Final elongation step*
	End at 4°C	*Reaction stopped*

Adjustments were made as required to the PCR mix and programme to enhance individual reactions. Primers were designed using *Primer 3* (Rozen, S. & Skaletsky, H. J., 2000).

2.6.3 Agarose gel electrophoresis

In order to visualise PCR results, reaction products were loaded on an agarose gel (agarose in 0.5 X TBE buffer). The percentage of agarose in the gel depended on the size of band to be resolved. The majority of band sizes fell between 150 bp and 250 bp, for which a 3% gel was used. Gels were run in 0.5 X TBE (4mM Tris.borate, 1 mM EDTA) buffer at 150 mV.

2.7 Populations used in study

2.7.1 Recombinant Inbred Line Populations

The inbred accessions, Bay-0, which originated from Germany, and Shahdara, from Tajikistan, along with the Bay-0 x Shahdara (Bay x Sha) recombinant inbred line (RIL) population were used in QTL analyses of root and rosette growth rate and petal

size. This RIL population was created, genotyped and provided by Olivier Loudet (Loudet, O. *et al.*, 2002). The Landsberg *erecta* x Columbia (L*er* x Col) RIL population (Alonso-Blanco, C. *et al.*, 1998) was also used in QTL analysis of rosette RGR and petal size; plants and seeds of this RIL population were kindly donated by Catherine Kidner of the University of Edinburgh. The Col and L*er* accessions both originated from Laibach in Germany, with the L*er* strain having undergone irradiation (Nottingham Arabidopsis Stock Centre online information; http://seeds.nottingham.ac.uk). Col and L*er* are two commonly used laboratory strains, L*er* being of particular interest in some studies because of the presence of a mutation in the *ERECTA* locus. Genotypes of the L*er* x Col RILs were taken from the Natural project (www.natural-eu.org).

2.7.2 Heterogeneous Inbred Families (HIFs)

A selection of HIFs were received from Olivier Loudet and analysed for the presence of QTL for root or rosette growth rate (see Table 2.2). The production and use of HIFs are further described in Chapter 6 (The Use of HIFs to Confirm QTL).

2.7.3 Stepped Aligned inbred Recombinant Strains (STAIRS)

Landsberg *erecta* x Columbia STAIRS for chromosome 2 (Koumproglou, R. *et al.*, 2002; see Figure 2.3), were used in analyses of petal size, root growth and rosette RGR. Seeds were received from Catherine Kidner of the University of Edinburgh. Pairs of STAIRS were analysed by *t*-test for the effect of an increase or decrease in the extent of the L*er* introgression.

HIF identification (= parental RIL number)	Segregating Marker (Chromosome)
90	MSAT 4.18 (4)
194	MSAT 1.10 and NGA248 (1)
397	NGA248 (1)
338	NGA172 and ATHCHIB2 (3)
209	MSAT 4.35 and MSAT 4.15 (4)

Table 2.2. HIFs analysed for QTL effects and segregating markers.

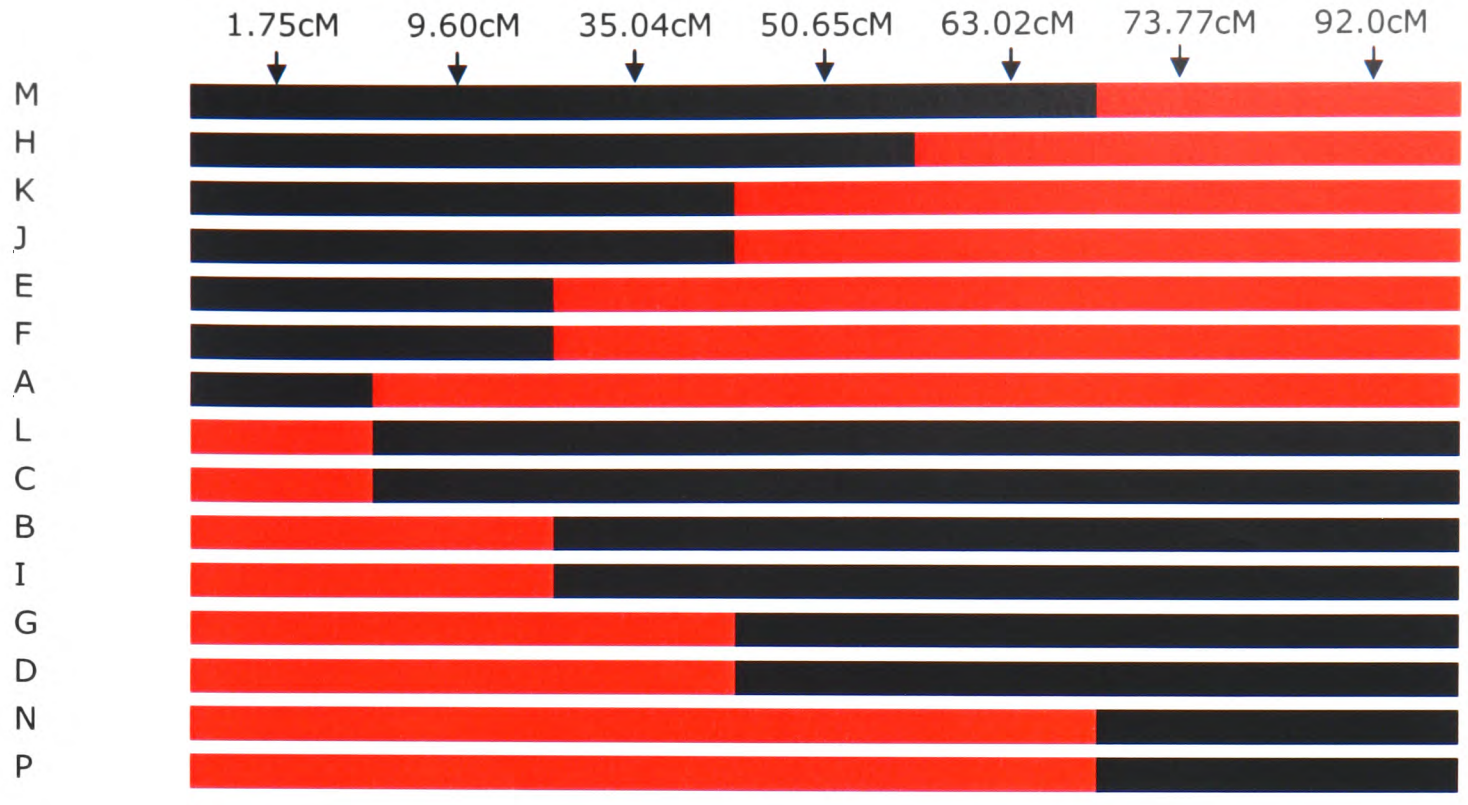


Figure 2.3. Landsberg *erecta* x Columbia STAIRS.
Chromosome 2 of L*er* x Col STAIRS (A-P) indicating Columbia DNA (black) with Landsberg DNA (red) introgressions and positions of the seven markers used for genotyping. The background genotype is Columbia.

A summary of all experiments carried out during these investigations is included in Table 2.3, below.

Experiment (Results section)	Dates carried out	Environmental Conditions and growth area	Experimental Design	Replications	Genotypes used	Seed source and history
Root length variation (4.2 – 4.3)	A: 9/10/03 – 22/10/03 B and C: 14/11/03 – 25/11/03.	22° C; Constant light; A: 0.5 x MS B: 0.5 x Johnson C ATS media; single shelf in growth cabinet.	1 genotype per plate; plates shuffled daily.	10 seeds per genotype.	Seeds from set of 18 Bay x Sha RILs recommended for showing good genotypic variation.	Bay x Sha sample seeds stored in laboratory, originally from Arabidopsis stock centre.
Root growth QTL analysis (4.4)	16/1/04 until 16/2/04.	LD; 22° C; 0.5 x MS medium; single shelf in growth room.	12 seeds per plate; 2 RILs per plate; 4 overlapping blocks (parental genotypes as controls in each block); plates shuffled daily.	12 seeds per genotype.	Core-pop set of 165 Bay x Sha RILs.	Seeds sent from Versailles by O. Loudet. Received December 2003.
Early root growth by environment (4.5)	Various experiments between December 2005 and March 2006.	Various conditions; 0.5 x MS medium; single shelf in growth rooms.	Round plates, positioned vertically; seeds arranged in grid pattern.	Up to100 seeds per genotype per experiment (see appendix).	Shahdara; Columbia.	Seeds collected during previous experiments; stored at room temperature in dry environment.
Rosette RGR variation (5.2)	9/10/03 until 10/11/03 and 14/11/03 until 17/12/03.	SD; 22° C; across shelves in growth room.	3 plants per pot; pots randomised across trays; trays shuffled twice weekly.	20 plants per genotype.	Seeds from set of 18 Bay x Sha RILs recommended for showing good genotypic variation.	Bay x Sha sample seeds stored in laboratory, originally from Arabidopsis stock centre.
Rosette RGR QTL analysis (5.3)	12/6/04 until 30./7/04.	SD; 22° C; several shelves in growth room.	5 overlapping blocks (parental genotypes as controls in each block). One plant per pot; 5 x 12 pots per tray; genotypes randomised across trays and trays shuffled twice weekly.	12 plants per genotype.	Core-pop set of 165 plus 30 additional Bay x Sha RILs.	Seeds sent from Versailles by O. Loudet. Received December 2003.

Experiment (Results section)	Dates carried out	Environmental Conditions and growth area	Experimental Design	Replications	Genotypes used	Seed source and history
Leaf number QTL analysis (5.4)	As rosette RGR QTL analysis above.					
HIFs for root length (6.2)	Various experiments between 15/9/04 and 25/5/05.	LD; 22° C; single shelf in growth room	Seeds sieved; seeds of HIF pairs alternated across plates; plates shuffled daily.	At least 20 seeds per genotype for each experiment (see appendix).	HIFs selected from Bay x Sha RILs.	HIFs sent September 04 from Versailles by O. Loudet or created from RIL seeds collected during previous experiments.
HIFs for rosette RGR (6.2)	31/1/05 until 7/3/05.	SD; natural daylength extended by white bulbs to 9 hours; temperature varied; single shelf in glasshouse.	One plant per pot; pots shuffled twice weekly.	18 plants per genotype.		
Petal size QTL analysis (7.4)	10/6/05 until 15/7/05.	LD; natural daylength; temperature varied; single shelf in glasshouse.	All plants grown in a single experiment; 3rd flower onward sampled; one plant per pot; pots shuffled twice weekly.	3 plants per genotype (about 4 flowers sampled per plant).	*Ler* x Col RILs.	*Ler* x Col RIL seeds received from C. Kidner (bulked in 2000 and travelled from US).
Petal size QTL analysis (7.4)	January 2006 until March 2006.	SD; natural daylength; temperature varied; single shelf in glasshouse.	All plants grown in a single experiment; 3rd flower onward sampled; one plant per pot; pots shuffled twice weekly.	2-5 plants per genotype.	Bay x Sha RILs.	Bay x Sha RIL seeds bulked in July 2004 after RGR QTL analysis.

Experiment (Results section)	Dates carried out	Environmental Conditions and growth area	Experimental Design	Replications	Genotypes used	Seed source and history
STAIRS – Petal size (8.2)	Various experiments between August and November 2005.	LD; 22° C; single shelf in growth room.	Comparisons were carried out within single experiments.	Various – see appendix.	*Ler* x Col STAIRS for Chromosome 2.	Seeds received from C. Kidner, previously from M. Kearsey.
STAIRS – Rosette RGR (8.3)	14/10/05 – 7/11/05.	SD; 22° C; across shelves in growth room.	Single experiment; one plant per pot; pots shuffled twice weekly.	12 plants per genotype.		
STAIRS – Root length (8.4)	14/10/05 – 24/10/05.	LD; 22° C; single shelf in growth room.	0.5 x MS medium; 10 seeds per plate; genotypes mixed across plates; plates shuffled daily.	10 seeds per genotype.		

Table 2.3. Summary of experiments.

3 Measuring Growth Rates

This chapter describes the development of measurement methods for the analyses of growth rates in rosettes, roots and petals of *A. thaliana*.

3.1 Rosette Relative Growth Rate

3.1.1 The functional approach

Relative growth rate (RGR) is defined by Hunt (1978) as "the increase in plant material per unit of material per unit of time". To calculate RGR, Hunt describes the fitting of polynomials to the plot of the natural log of plant area [ln *(plant area)*] against time.

A first degree polynomial should be a good fit to the growth of single organs or young whole plants, when plant size is log transformed. This assumes that growth is exponential at these stages, as cells divide at regular time points and equal size. When these data are log transformed, a linear relationship of ln *(number of cells)* vs. time is produced. To this plot, a first order polynomial is fitted:

$$\ln C = a + bT \qquad \textit{equation 3.1a}$$

(C = cell number; T = time)

RGR is deduced by differentiating this regression equation:

$$\frac{d \ln C}{dT} = b \qquad \textit{equation 3.1b}$$

$$\left(\frac{d \ln C}{dT} = \text{RGR}\right)$$

For a first-degree polynomial fit, RGR will be constant; in other words, RGR is assumed to be the same at any given time point. Alternatively, if the data are better suited, a second degree (quadratic) polynomial could be fitted:

$$\ln C = a + bT + cT^2 \qquad \textit{equation 3.2a}$$

In this case, differentiation gives the equation:

$$\frac{d \ln C}{dT} = b + cT \qquad \textit{equation 3.2b}$$

In such a case, changes in RGR over time are accounted for and instantaneous RGR can be calculated at each time point. Cell number may be substituted by leaf or rosette area in the above equations, assuming that cells are of an even size.

The more complex the degree of polynomial fitted to the data; the more complex further analysis will be. Hence, Hunt (1978) advocates the fitting of a first-order polynomial onto data that are close to, but not exactly, linear, in order that a single RGR value can be calculated for comparative analyses.

3.1.2 The classical and combined approaches

The above method of RGR calculation is known as the functional approach, as it fits a mathematical function to the collected data. An alternative method, known as the classical approach, involves calculation of size increase between every two harvests, followed by the plotting of these instantaneous growth rates against time for the course of the experiment. Poorter (Poorter, H., 1989) assesses the limitations of the classical and functional approaches to measuring plant RGR and suggests a new combined approach that retains advantages of both methods. By taking snapshots of RGR between pairs of harvests, the classical approach assumes constancy of RGR between time points; the functional approach, by fitting a polynomial across the curve of the experiment can allow for continuous changes of RGR over time. The RGR equation obtained by the functional approach is, however, very much influenced by the degree of polynomial chosen. This can result in either an under-fitting – by choosing too low a degree of polynomial – or an over-fitting – by choosing too high a degree of polynomial – of the function. It has also been found that the functional approach can fit very different RGR equations depending on the number of time points used in the experiment (Poorter, H., 1989).

Poorter's combined approach calculates RGR at intervals according to the classical approach, but uses overlapping intervals; thus harvests 1 and 3 are compared, 2 and 4 etc. The RGR data obtained are then smoothed by fitting a polynomial similar to the

functional approach, again fitting the simplest polynomial that adequately describes the data.

The combined approach presented by Poorter does appear to give a more accurate representation of RGR than would be given by a simple function fitted to growth data, whilst smoothing out the random fluctuations which remain in the classical approach. The resulting smooth curves for RGR over time are less complex than the time course of RGR obtained with the classical approach but more complex than the equivalent polynomial of the functional approach. However, although Poorter's combined approach might give a better representation of RGR over time, it may still be appropriate, in some cases, to fit a functional regression line for means of further analyses.

The following experiments used a simple functional approach, in order to obtain a single RGR value that can then be used for QTL analysis. This was adequate for initial mapping. Fitting Poorter's combined approach to the large number of RILs required for QTL analysis would have been highly time consuming and would have added considerable complexity to the QTL analysis. However, it may be useful for the further study of individual RILs to adopt the combined approach.

3.1.3 Measurement of rosette area

Traditionally, growth rate has been studied by means of destructive analysis of plants. Large populations are required so that at various time points a sample of plants can be removed and measured. Statistical analysis of the collected data gives an average plant growth rate for the population. For these analyses, however, I have developed a non-destructive method of analysis for the determination of growth rate of individual plants. Thus, a specific RGR value is obtained for each individual plant, rather than a mean population measure. This increases the efficiency of the study by reducing the materials and space required, as smaller sample numbers may be used than in a destructive analysis.

Leister *et al* (1999) demonstrated the effectiveness of non-destructive growth rate analysis in *A. thaliana*. In his method he took aerial photographs of *A. thaliana*

rosettes and measured the surface area of the whole of the rosette as an indication of plant size. *A. thaliana* plant architecture makes it suited to this method of analysis because the main vegetative growth stage is maintained in a flat rosette of leaves. Rosette area measurements were compared with fresh and dry weights measured for plants at the same growth stage under identical conditions. The correlation between weight and area was good for the early part of growth, but decreased after plants reached a fresh weight of 0.1g, due to leaf overlap causing an under-estimate of rosette area (Leister, D. *et al.*, 1999).

So, this method is mainly advantageous for the early stages of plant growth. It was also found that measuring the plant growth rate of individual plants by this method significantly decreased the coefficient of variation as compared to calculating an average growth rate from combined plant measurements (Leister, D. *et al.*, 1999). Thus, this method presents advantages over destructive methods of plant growth analysis because of the high statistical accuracy and the consequent reduction in sample number required.

In order to calculate the RGR of individual plants for QTL analysis, this simple, non-destructive method of measurement was adopted, which can be repeated any number of times during the vegetative growth of the plant. Aerial photographs were taken of the plants twice weekly using a digital camera. The photographs were modified in Photoshop (Adobe Systems Incorporated, 2004) to select only the green plant areas and eliminate the background; rosette surface areas were then measured in Image Tool (Wilcox, D. *et al.*, 1995),as described in Materials and Methods 2.3.

To show that rosette area could be used as a reliable approximation of plant growth, plants were harvested at 32 days after stratification and rosette area was regressed against plant fresh and dry mass. As in the experiments of Leister *et al* (1999), regressions showed a good fit (see Figure 3.1), with area approximating to fresh and dry mass with 89% and 96% accuracy, respectively, indicating that these non-destructive measurements are a good approximation for plant growth.

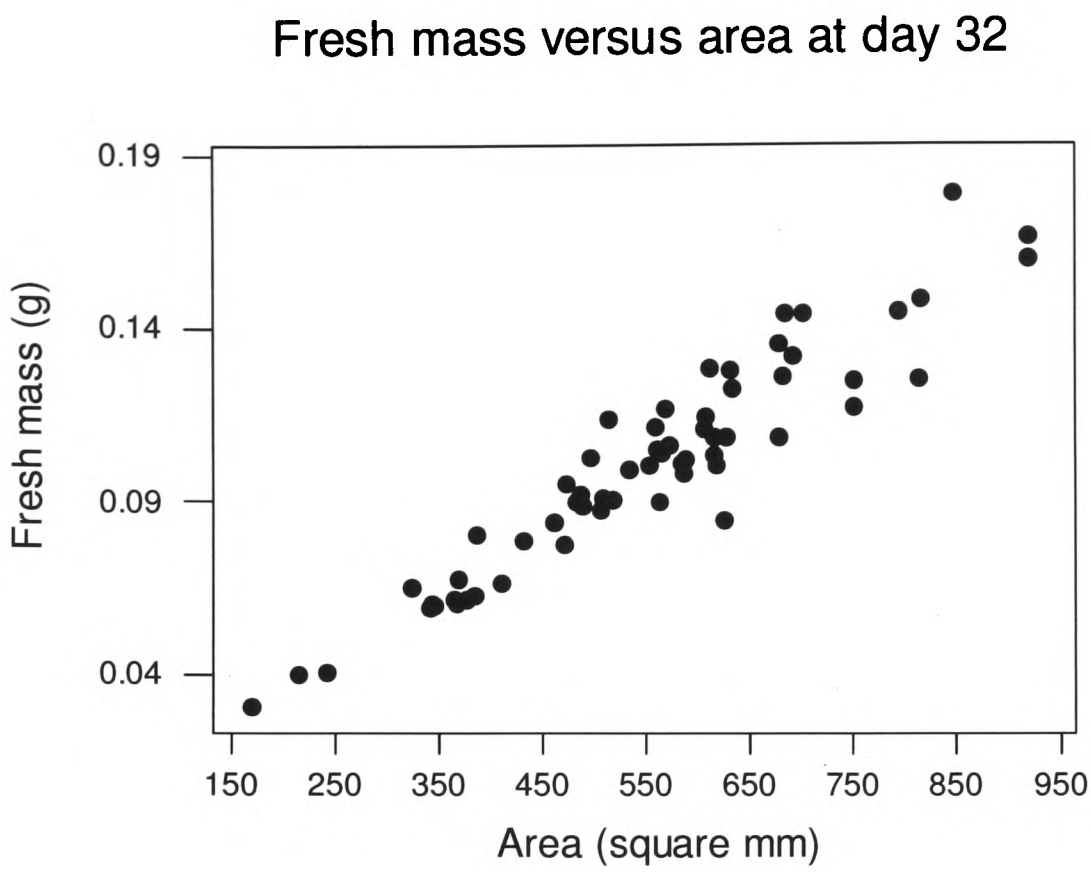


Figure 3.1. Regression of fresh mass against plant rosette area at 32 days. Showing good correlation of these two measurements. (Coefficient of determination (r^2) = 0.89) For dry mass against rosette area, r^2 = 0.96. Total plant number = 119.

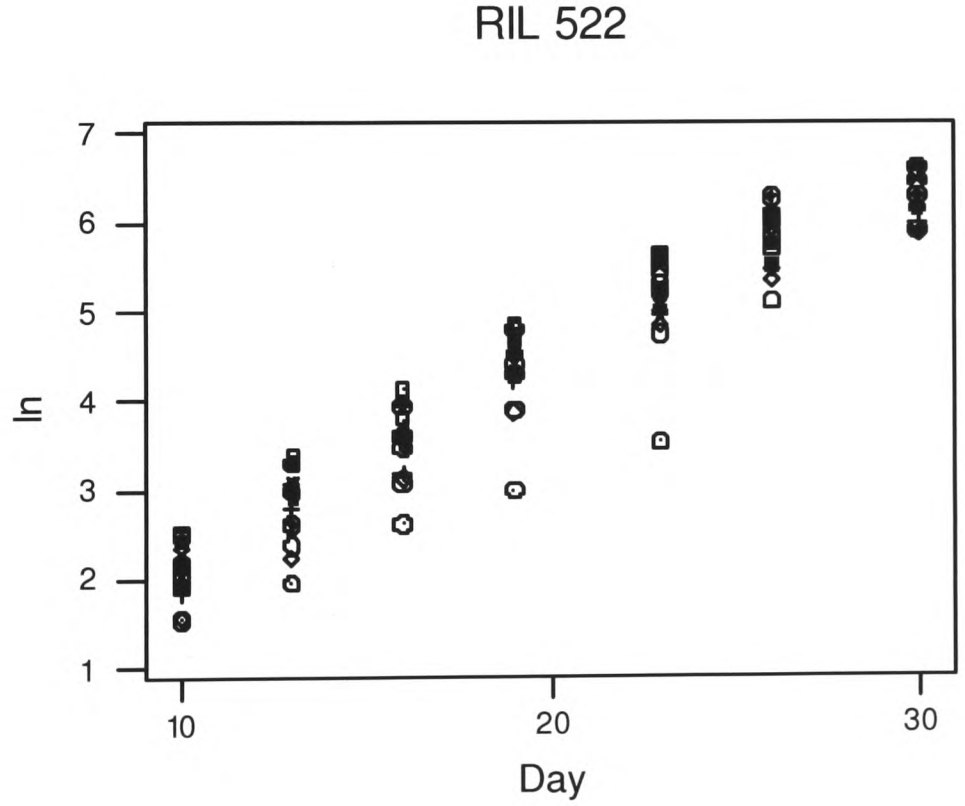


Figure 3.2. Calculating RGR
Example of regression of ln (*plant area*) against time for calculation of RGR, which is the slope of this regression.

Rosette surface areas were logged and simple linear regressions were fitted to the plots of ln*(plant area)* against time to obtain RGR for each plant (Equations 3.2a and 3.2b; see Figure 3.2). For QTL analysis, RGR was calculated for individual plants. A further benefit of calculating individual RGRs is that it allows analysis of the variance within each genotype, from which an approximation of environmental variance can be made and hence broadsense heritability can be estimated. From these data, the mean RGR for each genotype was calculated for use in the QTL analysis.

3.1.4 Leaf Overlaps

One bias that might arise from these growth measurements is an underestimate of plant area due to leaf overlaps. To investigate whether overlapping leaves were disrupting growth measurements, the leaf overlap for a sample of plants was estimated by measuring the areas on aerial photographs that appear to have two parts of a plant overlapping. The overlapping areas of six Bay-0 plants and six Shahdara plants were estimated and used to correct the original rosette area measurements. These corrected (plus overlap) areas were then compared with original (minus overlap) areas by means of a t-test. This analysis was repeated at progressive time intervals, as leaf overlap increases as plants mature. At day 14, the corrected measurements were not significantly different from non-corrected measurements (T-value = 2.09, p-value = 0.091). The difference between corrected and non-corrected values gradually increased with time, showing a significant difference by day 25 in Bay-0 (T-value = 4.72, p-value = 0.005) and day 28 in Shahdara (T-value = 4.58, p-value = 0.006). This agreed with Leister *et al* (1999) who found for Col-0 and L*er* accessions that leaf overlap did not affect the correlation of plant area with plant mass in the early stages of growth. The delayed appearance of significance in Shahdara correlated with Shahdara having the slower growth rate of the two parental genotypes.

Correcting for leaf overlap on an individual plant basis would have been highly time-consuming and would have risked additional errors caused by multiple estimations. An alternative was to estimate the mean overlap error for each genotype at the

significant time points and to correct individuals by the genotypic mean. Eighteen Bay-0 and seventeen Shahdara individuals were corrected for area at 25 and 28 days using the mean overlap errors calculated from the previous samples. These new values were used to calculate RGR, showing that there was a small but significant difference between corrected and non-corrected RGRs for both genotypes (see Figure 3.3). This alteration slightly decreased the difference between Bay-0 and Shahdara RGRs whilst slightly increasing the F-statistic obtained in the t-test of corrected values. However these changes were marginal.

In conclusion to the analysis of leaf overlaps, it was decided that the additional time required, plus the risk of introducing additional errors during estimation of overlaps, outweighed the slight increase in accuracy acheived by correcting RGR to account for leaf overlap. Therefore, in subsequent analyses, leaf overlaps were not taken into account, but measurements were stopped around the 24th day, to avoid the stage of growth in which leaf overlap would become a significant error.

One-way ANOVA: Sha corrected, Bay-0 corrected

```
Analysis of Variance
Source     DF        SS         MS         F        P
Factor      1  0.017874  0.017874    169.32    0.000
Error      33  0.003484  0.000106
Total      34  0.021357

                                  Individual 95% CIs For Mean
                                  Based on Pooled StDev
Level       N      Mean     StDev  ----+---------+---------+---------+--
Sha corr   17   0.19421   0.01324  (--*---)
Bay-0 co   18   0.23942   0.00633                              (---*--)
                                  ----+---------+---------+---------+--
Pooled StDev =  0.01027            0.195     0.210     0.225     0.240
```

One-way ANOVA: Sha non-corrected, Bay-0 non-corrected

```
Analysis of Variance
Source     DF        SS         MS        F        P
Factor      1  0.020030  0.020030   156.27    0.000
Error      33  0.004230  0.000128
Total      34  0.024260

                                  Individual 95% CIs For Mean
                                  Based on Pooled StDev
Level       N      Mean     StDev  -----+---------+---------+---------+-
Sha non-   17   0.19061   0.01482   (--*---)
Bay-0 no   18   0.23847   0.00649                               (--*--)
                                  -----+---------+---------+---------+-
Pooled StDev =  0.01132             0.192     0.208     0.224     0.240
```

Figure 3.3. Anova of Bay-0 and Shahdara rosette RGRs.
Corrected (above; Bay-0 – Shahdara RGR = 0.045) and non-corrected (below; Bay-0 – Shahdara RGR = 0.048) values.

3.2 Measurement of Root Growth Rate

In order to calculate root growth rate, only the primary root was considered over the early period of growth. Roots were also measured in a non-destructive manner, so that growth rate of the primary root could be calculated for individual plants over the measured time. Seeds were sown on square plates as described in Materials and Methods 2.2.1. Plates were placed near-vertically in the growth room so that the primary root would grow down the surface of the agar. The position of the root tip was marked on the back of the plate with a razor blade from the 3rd day after transfer to the growth room until the 12th day. Marking and measuring from the position of the root tip on the 3rd day of growth ensured that any differences in root length were not due to variability in germination times of the seeds.

After the 12th day, plates were photographed and the length of root growth from the 3rd to the 12th day of vertical growth was measured using Image Tool (Wilcox, D. *et al.*, 1995).

Root length was plotted against time and either a simple or a quadratic regression was chosen, according to which method gave the best fit for the data – by minimising the square of the residuals. This was seen to vary between experiments. When the data fitted a simple regression, i.e. there was no significant acceleration or deceleration of growth rate over time, a single measure of growth rate was calculated by dividing the distance grown over the number of days. This was equivalent to basing calculations on a single measure of root length achieved in the recorded period. When a quadratic regression gave the better fit, root growth rate was found to be accelerating during the course of the experiment. The quadratic equation,

$$L = a + bT + cT^2 \qquad \textit{equation 3.3a}$$

L = root length; T = time

was differentiated to give an equation for growth rate at each point of the experiment:

$$\frac{dL}{dT} = b + cT \qquad \textit{equation 3.3b}$$

$\frac{dL}{dT}$ = growth rate; T = time

Thus, instantaneous growth rates could be compared between genotypes at time points chosen throughout the experiment.

For the purposes of QTL mapping, a simple mean root length measurement for each RIL was used (equivalent to a simple constant growth rate over the course of the experiment). A quadratic equation was employed in subsequent analyses and growth rates were compared between genotypes at various time points.

3.3 Increasing Experimental Power by Reducing Environmental Variation in Growth Experiments

The power of QTL analyses depends on the heritability of the trait being measured, which can be estimated as

$$h^2 = \frac{V_G}{V_P} \qquad \textit{equation 3.4a}$$

where h^2 is the broad-sense heritability of the trait, V_G is the genetic variance and V_P is the phenotypic variance. V_P can be measured as the total variance in the measured trait over the population during the experiment. V_G can be calculated by subtracting environmental variance (V_E) from the phenotypic variance:

$$V_G = V_P - V_E \qquad \textit{equation 3.4b}$$

where V_E is estimated as the mean within-genotype variance (i.e. the variance that remains when there is no genetic variance between individuals).

During preliminary experiments to measure rosette RGR, broad-sense heritability was estimated at only 26%, which would lead to a low power of QTL analysis. This was enhanced in subsequent experiments by reducing V_E by increasing the consistency of the environment across the experiment. A single area of the

greenhouse was selected, over which light intensity was found to be most constant. Watering was carried out by standing pots in water until it could be seen to have soaked up to the surface of the soil: this encourages an even level of watering between individuals. The disadvantage of this method was that plants tended to be over-watered, encouraging some algal growth on the soil surface and increasing risk of infestations of pests such as sciarid flies. Plants were grown in individual pots at a distance sufficient to avoid competition effects between neighbours and were shuffled regularly during the experiment to minimise potential effects of local environmental differences. Finally, in order to reduce possible maternal effects, seeds were sieved twice to remove excessively large (those that would not go through a 0.14 mm^2 sieve opening) or small (those that would go through a 0.05 mm^2 sieve opening) seeds from each genotype before sowing (sieves were obtained from Sigma Chemical Company, P.O. Box 14508 St Louis, MO, 63178 USA). This removed additional variance in germination and early growth rate due to differences in seed size. Comparison of rosette RGRs of plants originating from small, medium or large seeds of the Bay-0 genotype by t-test, showed a significant difference between RGRs from small and medium seed size ($p<0.001$) and a marginally significant difference between large and medium seed size ($p<0.05$).

Environmental variance was similarly reduced during root growth experiments by thorough mixing of the media, use of a stable environment, shuffling plates throughout the course of the experiment and sieving seeds to reduce size variation.

During the QTL analyses, heritability for both rosette RGR and root growth rate was estimated at 43%.

3.4 Discussion

A non-destructive method of plant growth analysis was developed, allowing the calculation of rosette RGRs for individual plants. This results in a reduced sample number requirement compared to traditional destructive methods of analysis, allowing for the analysis of a large number of RILs – required for a QTL analysis – whilst maintaining a reasonably low total sample number. Development of this

method showed that total rosette area correlated with both fresh and dry mass and is therefore a feasible alternative estimate of plant size. Leaf overlaps were considered, as these might have artificially reduced rosette area measurements. It was concluded that overlaps only significantly affected measurements in the later stages of vegetative growth – that is towards the end of the fourth week. As the time required to correct for leaf overlap is considerable, and the corrections themselves may bring an additional amount of error in estimation, it was concluded that measurements should be stopped around the 24th day (at this rate of growth) and that no leaf overlap corrections should be made. This correlated with a previous study, which showed that leaf overlap did not affect rosette area measurements in the early stages of growth (Leister, D. *et al.*, 1999).

Additionally, a simple method of measuring early root growth rate was developed by germinating seeds on agar plates, positioned near-vertically so that root growth would progress linearly. Again, this method is non-destructive, so a smaller sample number is required to produce accurate results than in a destructive method of analysis. This experimental procedure is similar to that used by Loudet *et al* (2005) in their measurements of primary root length in the Bay-0 x Shahdara RIL population, but with the added precaution of measuring only from the third day of growth, in order to avoid the effects of variation in germination timing and early radicle extension rates.

Heritabilities of both traits were estimated and enhanced by means of reducing the environmental variance in the experiments. This resulted in an estimated broad-sense heritability of 43% during the QTL analysis for both rosette RGR and root growth rate. This represents a reasonable heritability for the detection of moderate-effect QTL. Previous studies have identified QTL with trait heritability ranging from 0.36 to 0.69 (Juenger, T. *et al.*, 2000; Juenger, T. *et al.*, 2005). However, if multiple small-effect QTL are present in the RIL population studied, each QTL will have a heritability of only a fraction of this total heritability. Therefore the actual heritability of an individual QTL will be small and this will reduce the power of the test (Kearsey, M. J. & Farquhar, A. G. L., 1998). The capacity to enhance heritability by reducing environmental variance suggests that genotypic variance can

be masked by variation in the environment. This may indicate that QTL interact plastically with environmental signals, thus reducing the power of QTL detection in non-uniform environments.

The following two chapters discuss the results of using these methods of measurment for QTL analysis of growth rate in roots and rosettes.

4 Root Growth Rate

4.1 Introduction

The plant root is essential for anchorage and the uptake of water and essential nutrients, making it a potentially important determinant of plant fitness. This is particularly the case in the early phase of seedling establishment, where the rate at which a plant can extend its root to utilise available nutrients will affect its ability to compete with neighbouring plants.

Primary root growth occurs mainly by unidirectional expansion and division of cells in the growth zone at the tip of the root. This growth zone can be divided into two areas: the meristem, situated just above the quiescent centre, where cells divide and expand; followed by the elongation zone where no division occurs but cells continue to expand (Beemster, G. T. S. *et al.*, 2003; Dolan, L. & Davies, J., 2004). The sizes of both these regions influence the rate of root growth. In the natural short-root accession, Umkirch-1, and the NIL, brx^{s} – created by introgression of the *BREVIS RADIX (BRX)* region of Umkirch-1 into the average root length accessioN, Slavice-0, – reduced primary root length is associated with a reduction in size of both the meristem and the elongation zone. Hence, there are fewer, shorter cells in Umkirch-1 and brx^{s} compared to Slavice-0 roots (Mouchel, C. F. *et al.*, 2004). Similarly, variation in root elongation rate in 18 natural accessions of *A. thaliana* correlated with variations in mature cell size (dependent on the rate and duration of elongation undergone by cells) and variations in cell number (a product of the rate and extent of cell division; Beemster, G. T. S. *et al.*, 2002). Therefore, root growth rates may be modified by variations in the intrinsic controls of meristem and elongation zone activity.

The extents of these two zones are thought to be dependent on auxin and cytokinin concentrations (Chavarria-Krauser, A. *et al.*, 2005), so may be affected by variations in the production, distribution and response to these phytohormones. As any length increase in root tissue requires the elongation of root cells, factors that limit cell

elongation, such as turgor pressure and cell wall extensibility (Pritchard, J., 1994) will also be important in regulating root growth rate.

Environmental responses in root growth include gravitropism, hydrotropism (Eapen, D. *et al.*, 2005) and responses to local nutrient availability (Lopez-Bucio, J. *et al.*, 2003). These responses affect the direction, locality and rate of root growth, thereby altering root architecture to best exploit the local environmental conditions. Hormonal signalling is likely to play a crucial role in controlling root growth. For example, the phytohormone auxin stimulates root growth by modulating the effect of giberellin on DELLA protein concentration in root cells, such that giberellin-deficient *A. thaliana* mutants have shorter roots in comparison to wild-type (Fu, X. & Harberd, N. P., 2003).

Variation in primary root length has been observed in natural accessions of *A. thaliana* (Beemster, G. T. S. *et al.*, 2002; Mouchel, C. F. *et al.*, 2004) and has led to the identification of at least one novel gene, *BRX* (Mouchel, C. F. *et al.*, 2004). A QTL analysis in the Bay-0 x Shahdara RIL population also identified 3 putative primary root length QTL, accounting for 5%, 7% and 14% of the variance observed in the RIL population (Loudet, O. *et al.*, 2005). The short-root allele of *BRX* is likely a mutation unique to the Umkirch-1 accession, as it was not found in closely related accessions Umkirch-2, -3 or -4 (Mouchel, C. F. *et al.*, 2004); but it is feasible that mutations in this locus could have arisen in other accessions and cause variations in root growth rate which would be identified in new QTL analyses, such as the one described in this chapter.

Also, this QTL analysis in the Bay x Sha RIL population may further validate the locations of primary root length QTL mapped by Loudet *et al* (2005). However, because root growth is highly influenced by environment, it is possible that QTL identified during one experiment will not be apparent in a QTL analysis of the same population under different conditions. Loudet *et al* (2005) measured primary root length after 9 days of near-vertical growth on basic Arabidopsis media with 1% sucrose and 0.6% agarose; whereas the conditions in this analysis are growth on 0.5 × MS medium with 0.6% sucrose and 1% agar, measuring vertical growth between

the third and twelfth days. Thus, the growth stage, position and sugar and nutrient composition vary between these two studies. A further difference between these experiments is that Loudet *et al* grew roots through the medium, by initiating growth on horizontal plates and then placing them in a near-vertical position after three days. In this analysis, plates are placed near-vertically before germination, so that roots grow down the surface of the medium. Although growth rate does not appear to be affected by the response of the primary root to touch stimuli (Massa, G. D. & Gilroy, S., 2003), it has been shown that primary root growth rate can be affected by the angle of the plate from the vertical (Buer, C. S. *et al.*, 2000), probably due to the changes in gravitropically-induced frictional forces experienced by the root against the medium. Frictional forces and the touch response will be increased in the roots grown by Loudet *et al* compared to surface-grown roots – factors which could affect the growth of the root and therefore the nature of QTL detected. Loudet *et al* argue that growth of the root through the medium was advantageous in avoiding the wave-like growth that is sometimes observed when roots are grown on the surface of a medium, however, sinusoidal patterns of root growth were rarely observed during this analysis. It was therefore decided that, for this analysis, surface growth of primary roots should be used, in order to minimise the detection of touch-stimulus and other QTL that may have a secondary-effect on growth rate.

Correlation with the QTL mapped by Loudet *et al.* (2005) would indicate that these QTL were not specific to the environmental conditions employed in one experiment and would act as further confirmation of the locations of such QTL. This analysis also has the potential to identify novel primary root growth QTL in the Bay x Sha RIL population, which did not significantly affect growth rate during previous experiments.

This chapter describes an analysis of natural root growth rate variation, an assessment of the effects of environmental variation and the mapping of QTL in the Bay x Sha RIL population.

4.2 Variation for root growth rate in the Bay-0 x Shahdara RIL population

An initial experiment was designed to look for root growth rate variation between RILs in the Bay x Sha RIL population, in order to assess the usefulness of this population for QTL analysis of root growth rate. Seeds for a set of 17 RILs, plus the parental genotypes were sterilised and plated on 0.5 × MS media (1% Agar, 0.6% sucrose). After three days of stratification, plates were placed near-vertically under constant light in a 22°C growth cabinet. Twenty samples of each RIL were sown onto a single plate. Root tip position was scored daily from the 3rd until the 12th day after stratification, whereupon root extensions per day were measured. A simple regression fitted well to the data of root length against time, implying that growth rate did not significantly change over the course of the experiment, so a single average root growth rate value was calculated.

Mean root growth rates for each RIL varied between 4.4 mm and 8.1 mm per day, with a pooled standard deviation of 0.47 mm. Analysis of variance (ANOVA) showed that this constituted significant variation ($p = 0.000$) and that there was a good spread of means across this sub-population of Bay x Sha RILs (see Figure 4.1).

4.3 Environmental effects on root growth rate

To consider whether QTL associated with nutrient capture, rather than intrinsic growth rate might be detected, a selection of the RILs used above was grown on ATS and 0.5 × Johnson media to assess the effect of changes in nutrient provision on root growth. The RILs chosen included the fastest and slowest growing roots from the 0.5 × MS experiment plus the parental genotypes. Genotypes were mixed across plates, such that ten seeds of each of six genotypes were sown, with one or two per plate (twelve seeds total per plate). Other environmental factors and the method of experiment were maintained.

One-way ANOVA: Bay-0, Sha, RIL 523, RIL 679, RIL 794, RIL 836, RIL 777, RIL 698,

Analysis of Variance

Source	DF	SS	MS	F	P
Factor	18	152.209	8.456	38.64	0.000
Error	171	37.422	0.219		
Total	189	189.631			

Level	N	Mean	StDev
Bay-0	10	5.0090	0.3426
Sha	10	6.1560	0.4115
RIL 523	10	7.1180	0.5234
RIL 679	10	6.1840	0.4308
RIL 794	10	5.4370	0.3491
RIL 836	10	5.5230	0.6132
RIL 777	10	4.7570	0.3379
RIL 698	10	8.1450	0.8365
RIL 677	10	4.7580	0.4468
RIL642	10	5.1460	0.3632
RIL666	10	5.8980	0.4539
RIL 641	10	6.0290	0.6371
RIL 881	10	4.5510	0.1879
RIL 514	10	4.7290	0.4489
RIL 522	10	4.4390	0.3188
RIL 604	10	5.8290	0.3745
RIL 598	10	5.3520	0.6734
RIL 600	10	6.0350	0.3904
RIL 537	10	5.6790	0.2683

Pooled StDev = 0.4678

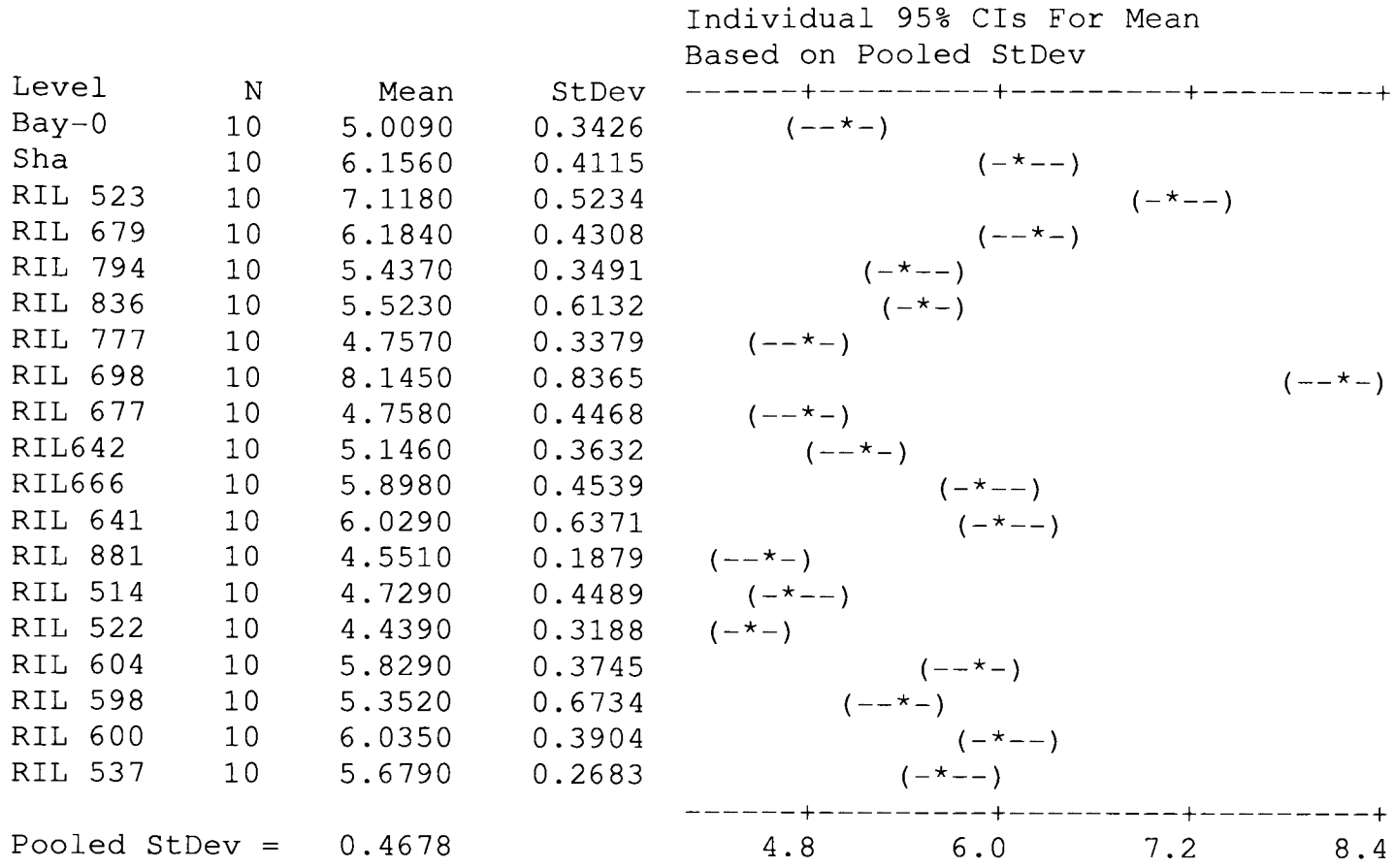


Figure 4.1. One-way ANOVA of root growth rate for 17 Bay-0 x Shahdara RILs plus parental genotypes.
Mean root growth rates, shown with 95% confidence intervals, show a good spread of variation amongst RILs, indicating that natural variation of root growth rate is present in this RIL population.

Under these conditions, root growth rate accelerated during the course of the experiment, thus producing a quadratic regression when root length was fitted against time. Media effects were analysed by calculating the root growth rate on the 10^{th} day for growth on ATS and 0.5 × Johnson media and comparing these with the average root growth per day on 0.5 × MS. Actual growth rates could not be compared directly, as these showed an overall variation between media (growth on ATS was generally faster than growth on 0.5 × Johnson on day ten). Instead, RILs were ranked in decreasing order of growth rate and the ranked data were compared (see Figure 4.2). Considerable changes in ranking were observed between growth media, indicating that the complexity of changes in nutritional environment affected the growth of different genotypes differently.

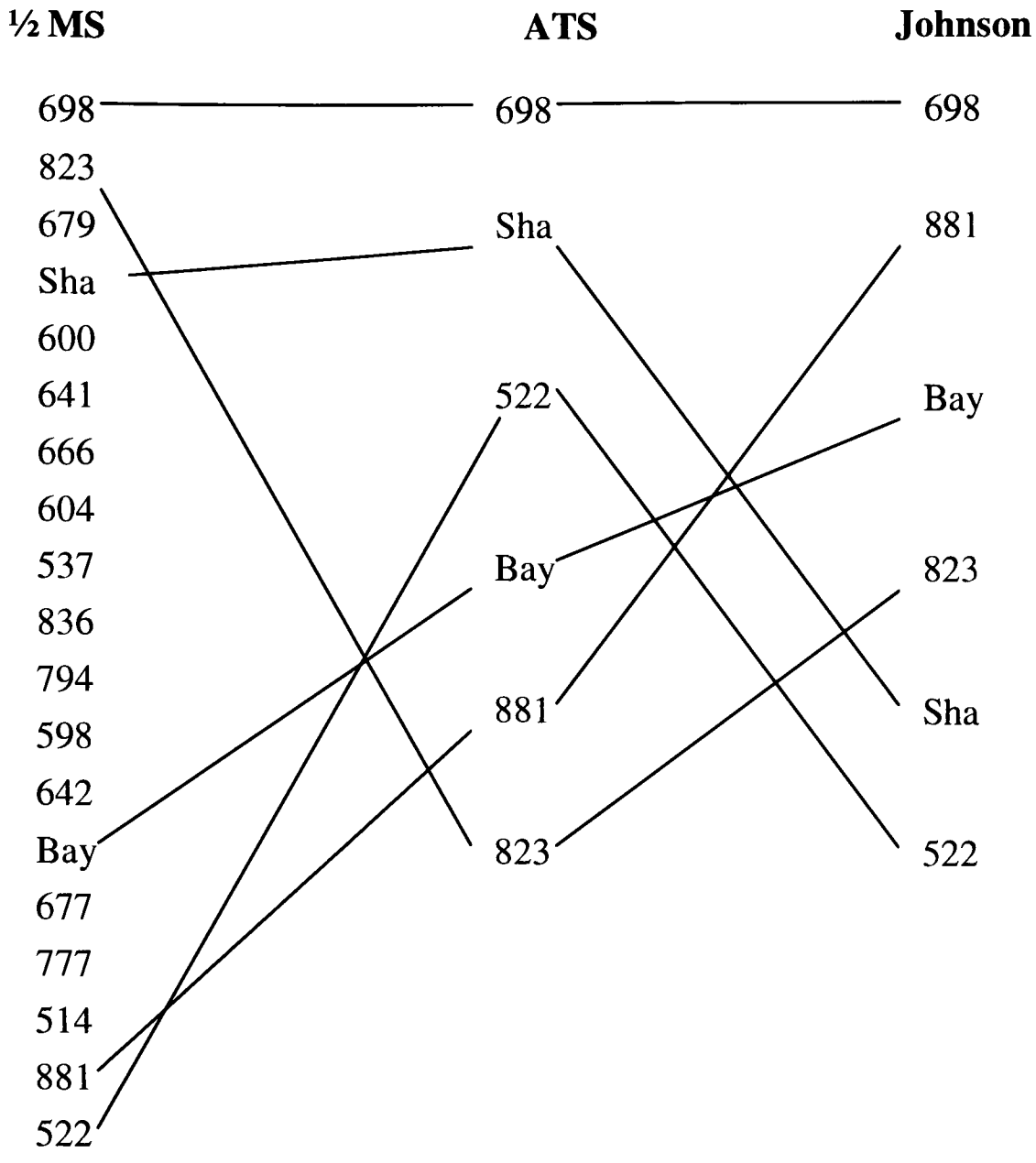


Figure 4.2. Comparison of ranked root growth rates when grown on 0.5 × MS, ATS or 0.5 × Johnson media.
Numbers identify RILs; parents Bay-0 (Bay) and Shahdara (Sha) are included in the analysis. Root growth rates were ranked from highest to lowest in each experiment for comparison between media. Straight lines connect genotypes which were present in more than one analysis. Plant numbers = 10 per genotype.

Position effect could also be analysed in this experiment, as genotypes were mixed across six plates of each medium. For each root, the difference between the individual initial growth rate and the mean initial growth rate for all roots of that line was calculated and values were compared between plates to assess whether any plate showed a tendency for faster or slower initial growth rate. This was repeated for acceleration of growth rate. No significant difference was found for the deviations from the mean of either initial growth rate or acceleration of growth rate (one-way ANOVA: $p = 0.33$ and $p = 0.34$, respectively). Additionally, position on the plate

was assessed for its effect on root growth rate. Initial growth rates and accelerations of growth rate were compared for all seeds growing in each of the positions 1 through 12, numbering from the left to the right side of the plate. Again, there was no significant effect of position on the plate observed for either initial growth rate or acceleration of growth rate (one-way ANOVA: $p = 0.173$ and $p = 0.821$, respectively).

However, it is possible that, over a larger experiment, plate position could contribute to environmental variance, so for future experiments a compromise was used of splitting each genotype between two plates, with six seeds of each genotype on each plate. Thus, any dramatic effect of a plate (perhaps due to an extreme position in the experiment, or an uneven mixing of nutrients in the medium) could be detected and the affected data removed from the analysis.

4.4 QTL analysis of root growth rate in the Bay-0 x Shahdara RIL population

QTL analysis for root growth rate was carried out on the sub-set of 165 Bay x Sha RILs. 12 seeds of each RIL were grown across two plates (two genotypes per plate) of 0.5 × MS (1% Agar, 0.6% Sucrose). Seeds were stratified for 3 days after sterilisation in EtOH and then grown in a near-vertical position in a long-day growth room. The experiment was carried out in four blocks with parental genotypes grown each time to monitor heterogeneity in the environment. Plates were mixed randomly across the shelf of the growth room and roots were scored from 3 days after stratification ("day 3") until 10 days after stratification ("day 10").

Quadratic regressions were seen to give a better fit to individual root growth values than linear regressions, indicating that there was some acceleration of growth rate over the course of the experiment. However, it was decided to use the full length values of root growth over the measured period for the QTL analysis. This was partly to avoid complications of varying growth rates over time and partly because the QTL analysis programme deals better with large, whole values than with the smaller figures required for growth rate. Comparison of the data by Pearson

Correlations showed that full length correlated most strongly with growth rate at day 10 (Pearson's Correlation = 0.910, p-value = 0.000) and less strongly with day 3 growth rate (Pearson's Correlation = 0.629, p-value = 0.000); i.e. the coefficients of determination were 0.82 and 0.40, respectively. Day 3 and Day 10 growth rates were weakly correlated (Pearson's Correlation = 0.277, p-value = 0.000).

Analysis of Bay-0 and Shahdara root growth over the course of the experiment showed that environmental differences contributed to changes in root growth both between blocks of the experiment and within a block: that is, there was environmental variation within the growth area over both time and space. An ANOVA of Bay-0 root lengths during the four blocks of the experiment gave a significant difference between blocks, with blocks BC and DE reaching a longer final length than blocks FG and HIA ($p = 0.005$; see Figure 4.3). Mean root length for Bay-0 plants varied between 33.8 mm (in the final block of the experiment) and 44.7 mm (in the first block). Shahdara roots did not show significant variation between blocks according to an ANOVA ($p = 0.307$; see Figure 4.3).

The pattern of Bay-0 and Shahdara root lengths across the blocks of experiment did not coincide. This suggests that either a further positional effect of Bay-0 plates against Shahdara plates was involved (as plates were not put in the same positions during each block of the experiment), or that Bay-0 and Shahdara genotypes responded differently to the environmental fluctuations that occurred. Unfortunately, this inconsistency of control root lengths ruled out a simple correction for root lengths across blocks. The data were therefore left untransformed, although individual outliers were removed from measurements within a RIL.

One-way Analysis of Variance: Bay-0 Full Root Length (Day 3 – Day 10)

```
Analysis of Variance
Source     DF        SS        MS        F        P
Factor      3     895.2     298.4     5.15    0.005
Error      36    2084.1      57.9
Total      39    2979.3

                                    Individual 95% CIs For Mean
                                    Based on Pooled StDev
Level       N      Mean     StDev  ---+---------+---------+---------+---
Bay BC     12    44.731     6.058                     (-------*------)
Bay DE     10    44.497    10.377                    (-------*-------)
Bay FG      9    36.966     7.346         (--------*-------)
Bay HIA     9    33.764     5.912   (-------*--------)
                                    ---+---------+---------+---------+---
Pooled StDev =    7.609             30.0       36.0       42.0       48.0
MTB > AOVOneway  'Sha BC' 'Sha DE' 'Sha FG' 'Sha HIA'.
```

One-way Analysis of Variance: Shahdara Full Root Length (Day 3 – Day 10)

```
Analysis of Variance
Source     DF        SS        MS        F        P
Factor      3     264.1      88.0     1.25    0.307
Error      35    2466.1      70.5
Total      38    2730.2

                                    Individual 95% CIs For Mean
                                    Based on Pooled StDev
Level       N      Mean     StDev  ----------+---------+---------+------
Sha BC     10    43.149    10.684        (---------*----------)
Sha DE     10    47.581     8.449                  (----------*----------)
Sha FG      9    44.466     6.231           (----------*----------)
Sha HIA    10    40.435     7.347  (----------*----------)
                                    ----------+---------+---------+------
Pooled StDev =    8.394                  40.0       45.0       50.0
MTB >
```

Figure 4.3. ANOVA of root lengths.
Showing (above) in the four blocks of the experiment, significant differences in mean Bay-0 root growth rates between blocks and (below) Shahdara root lengths in the same four blocks, which did not show significant differences between blocks.

Mean RIL root lengths spread either side of parental mean values (40 mm and 44 mm for Bay-0 and Shahdara, respectively) in a distribution approximating to the normal distribution (see Figure 4.4) , as did growth rates at day 3 and day 10. This suggested the presence of genetic variation within this RIL population.

Average standard deviation for root length within a RIL was 7.17 mm, compared to the standard deviation of RIL means across the experiment of 7.05 mm. This high within-line variation is probably a cumulative effect of environmental variation between plates and between positions within each plate and variation due to seed size differences.

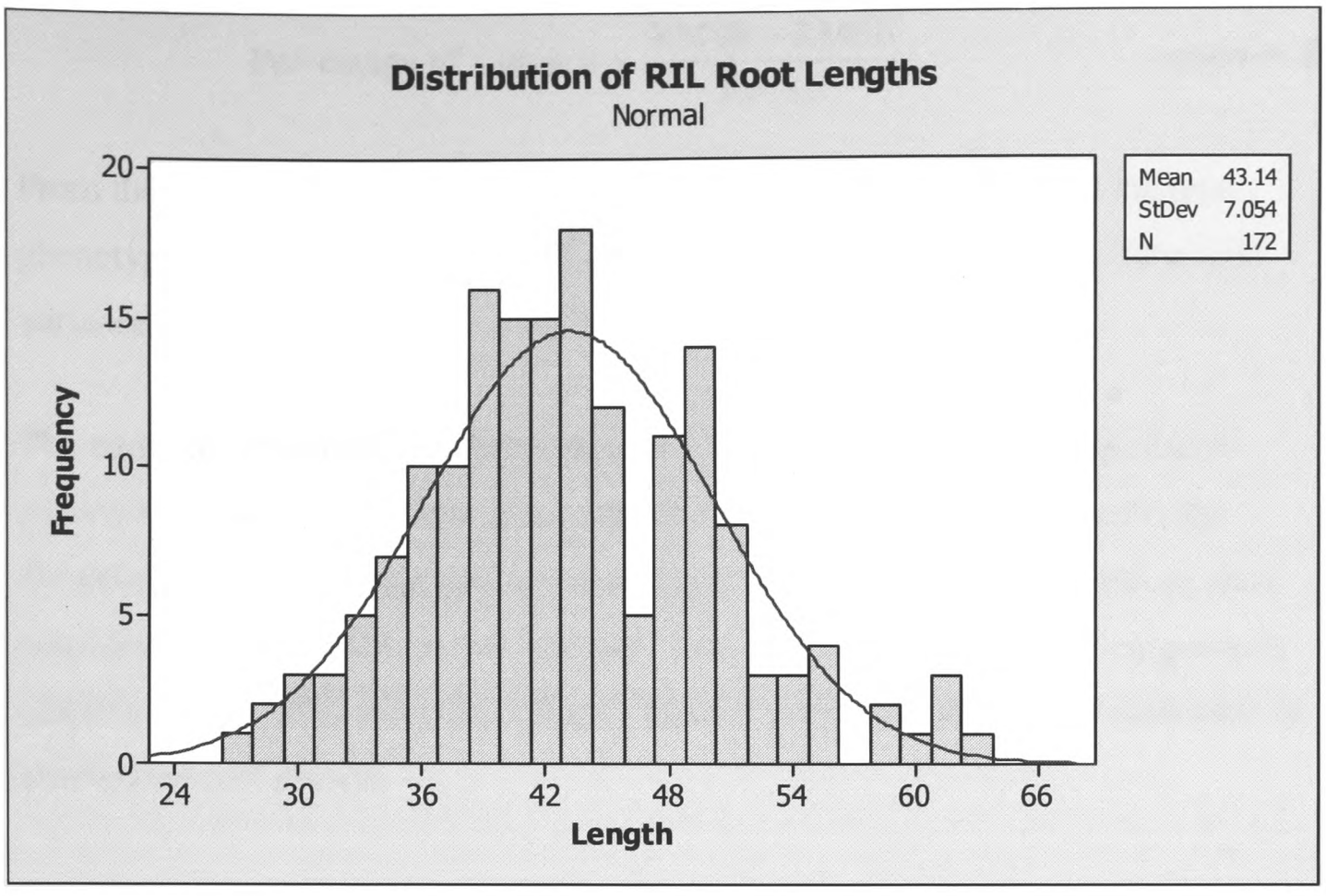


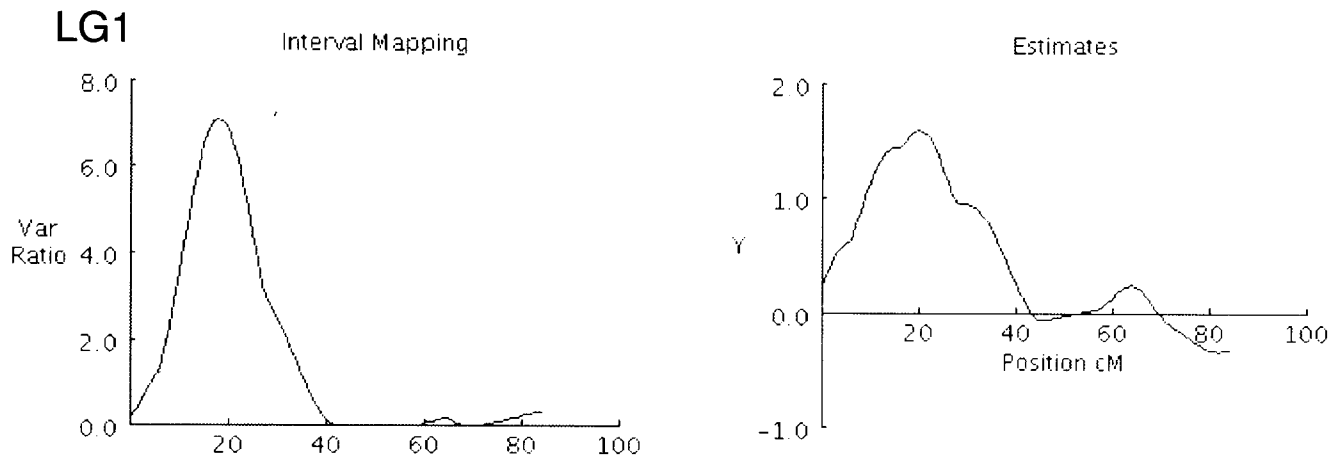
Figure 4.4. Distribution of RIL mean root lengths.
Showing approximation to normal distribution with mean 43.04 mm and standard deviation 7.05 mm. The number of samples per RIL varied from 2 to 12 with a median of 11; number of genotypes = 164.

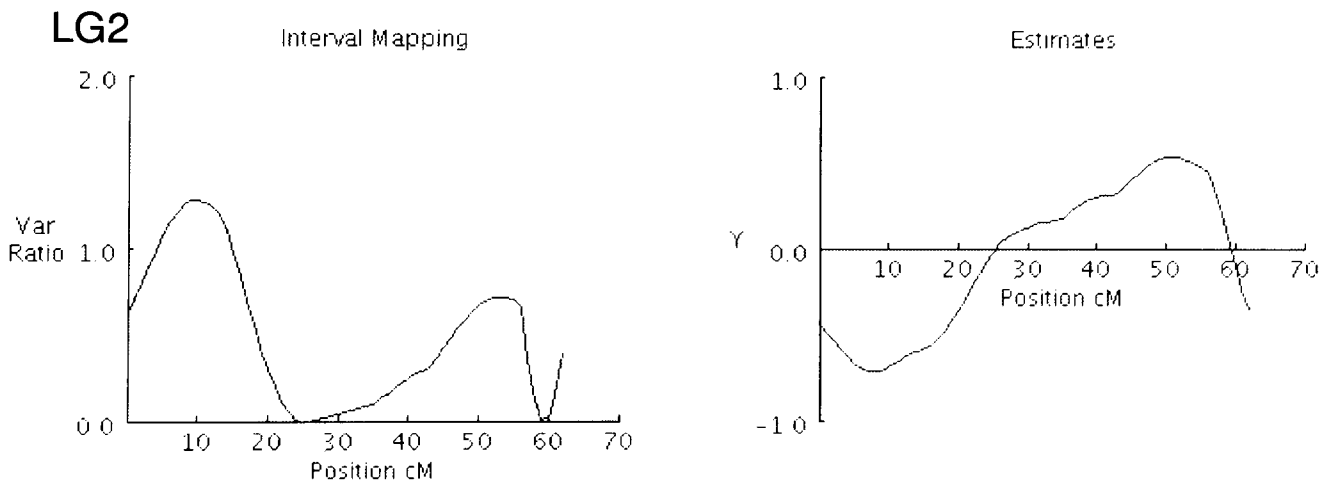
Mean root length from day 3 to day 12 was used for the QTL analysis. Experiment-wide permutations (x 1000) gave a threshold F-statistic of 11.2 ($p = 0.05$) or 14.5 ($p = 0.01$). Two significant QTL were predicted on this basis with F-statistics of 13.8 (linkage group 4, 28 cM) and 15.28 (linkage group 5, 44 cM; see Figure 4.5). Although the interval mapping variance graph for linkage group 4 had a very broad peak, the mapping programme was unable to separate this effect into two significant QTL. The estimated additive effects of the predicted QTL were -2.16 mm and -2.35 mm for those of chromosomes 4 and 5, respectively. The negative sign indicated that for both QTL the Shahdara alleles increased root growth rate. The residual mean sum of squares generated in regression by *QTL Express* for the reduced (RMSR; without QTL) and full (RMSF; with QTL) models of the data were used to calculate the percentage of variance contributed by each QTL (Knott, S. A., 2004):

$$\text{Percentage of variance} = \frac{\text{RMSR} - \text{RMSF}}{\text{RMSF}} \qquad \textit{equation 4.1}$$

From this calculation, the QTL on chromosome 4 accounted for 7% of the total phenotypic variance, whilst the QTL on chromosome 5 explained 8% of the total variance.

The variance ratio peaks on chromosomes 1 (at 18 cM) and 3 (at 55 cM) had F-statistics below the 95% significance thresholds of 7.11 and 7.54, respectively. However, these peaks appeared distinct despite their low significance and so were considered putative QTL in this analysis. Both of these putative QTL had positive additive effects, indicating that a Bay-0 allele at these positions would contribute to accelerated root growth.





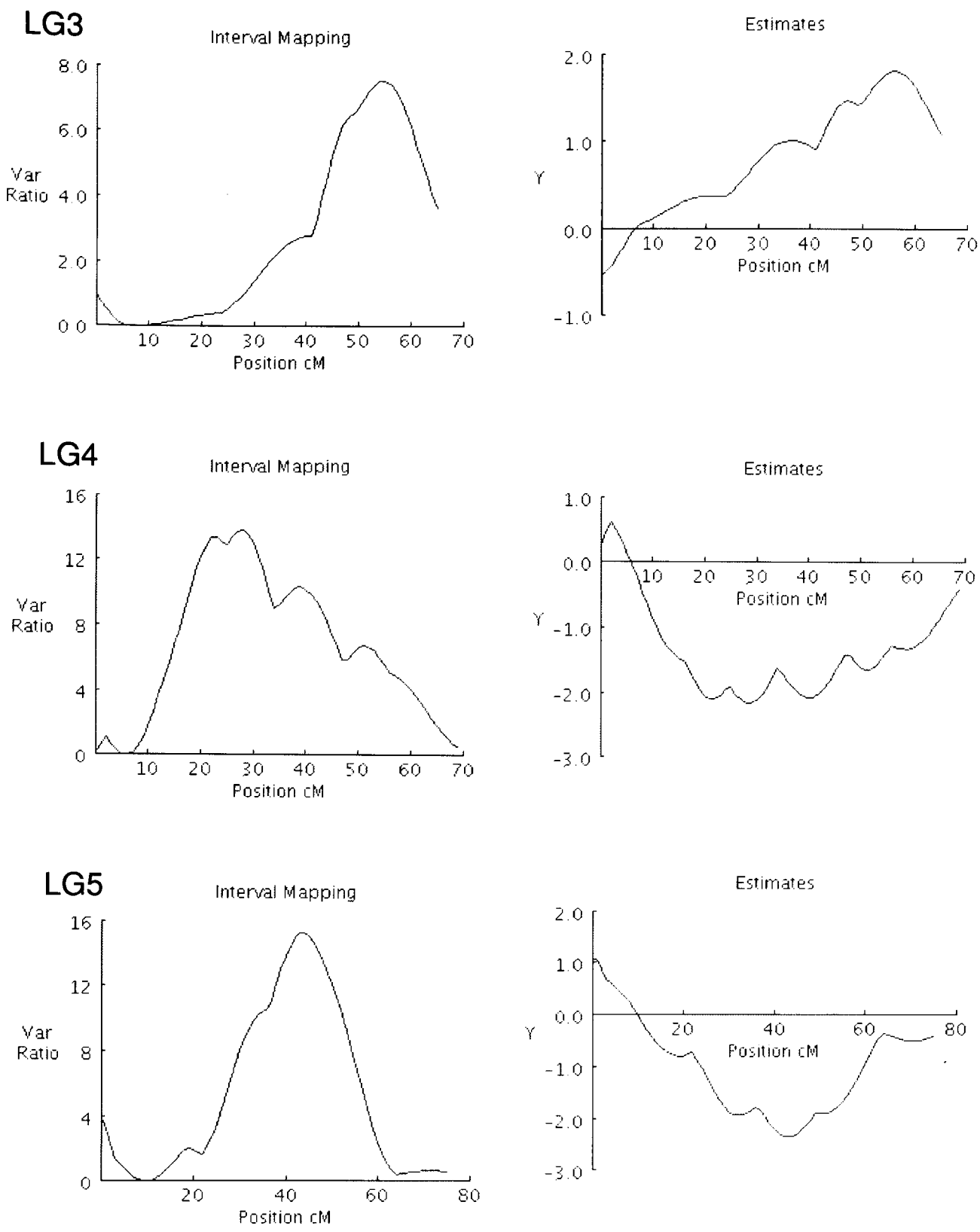


Figure 4.5. QTL Analysis of root growth in the Bay x Sha RIL population. *QTL Express* variance ratios (left) and estimates of additive effects (right) by interval mapping for the 5 linkage groups (LG1-LG5).

4.5 Genotype-environment Interactions in Early Root Growth

To assess whether phenotypic plasticity was likely to play an important part in these experiments, two genotypes were tested for their responses to changing environments. Columbia and Shahdara accessions were sterilised, stratified and

germinated under different light and temperature conditions. Growth of the radicle (early root) was measured 72 hours after seeds were placed in growth positions. Three effects were examined – temperature, light quality and light quantity.

4.5.1 Temperature effects on Columbia and Shahdara early root growth

Temperature can be difficult to control accurately across a wide experimental area, so plants were tested for a temperature-dependent plastic effect, which might affect the environmental variance in growth measurements. Seeds were germinated under three temperatures (16° C, 18° C and 22° C) whilst other environmental conditions were maintained. Temperature had a similar effect on the early root growth of both accessions, with an increase in temperature correlating with an increase in root growth over 72 hours (see Figure 4.6). Lengths of Shahdara roots were less than those of the Columbia accession at all temperatures, which may be due to delayed germination of Shahdara seeds, as was observed in preliminary experiments. So it appears that temperature has a similar effect on germination and early root growth in both accessions.

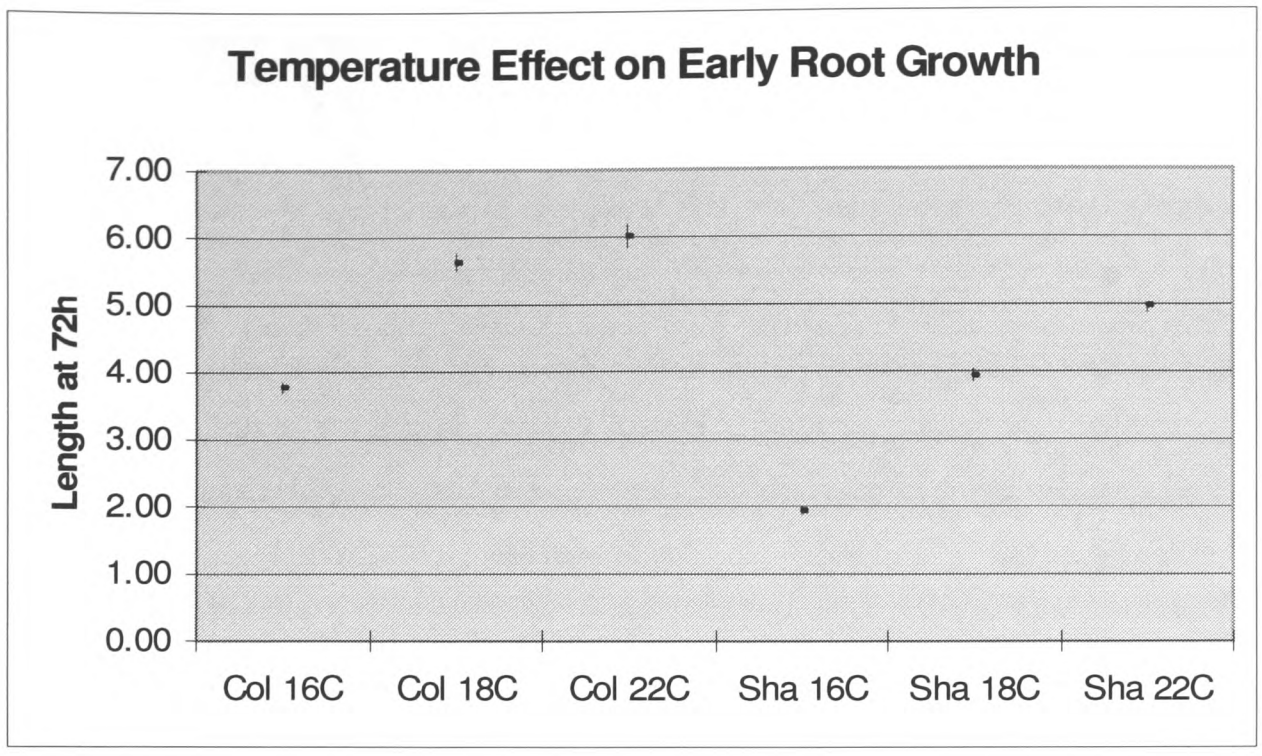


Figure 4.6. Temperature effect on early root growth.
Showing means and standard error for root length after 72 hours for Columbia (Col) and Shahdara (Sha) accessions at 16, 18 and 22° C. Whilst Shahdara growth is always reduced compared to Columbia, the two accessions show a similar gradient of response to the temperature increase. Plant numbers: about 100 plants per genotype per treatment.

4.5.2 Light quality effects on Columbia and Shahdara early root growth

To assess whether Columbia and Shahdara exhibited different responses to competition in germination and early root growth, seeds were germinated under two different light quality conditions. Reducing the red:far-red ratio, by the addition of far-red diodes, mimics the competitive effect of near-growing plants. A genotype-dependent difference in early response to competition would be relevant for seedlings germinated in close proximity, such as on agar plates. Full light conditions were compared with low red:far-red light, by the addition of far-red diodes in the latter experiment. Neither accession showed a significant response to the change in light quality during the first 72 hours of root growth (See Figure 4.7). Unpaired t-tests of growth in full light versus growth in low red:far-red light gave p-values of 0.175 and 0.339 for Columbia and Shahdara radicle lengths, respectively.

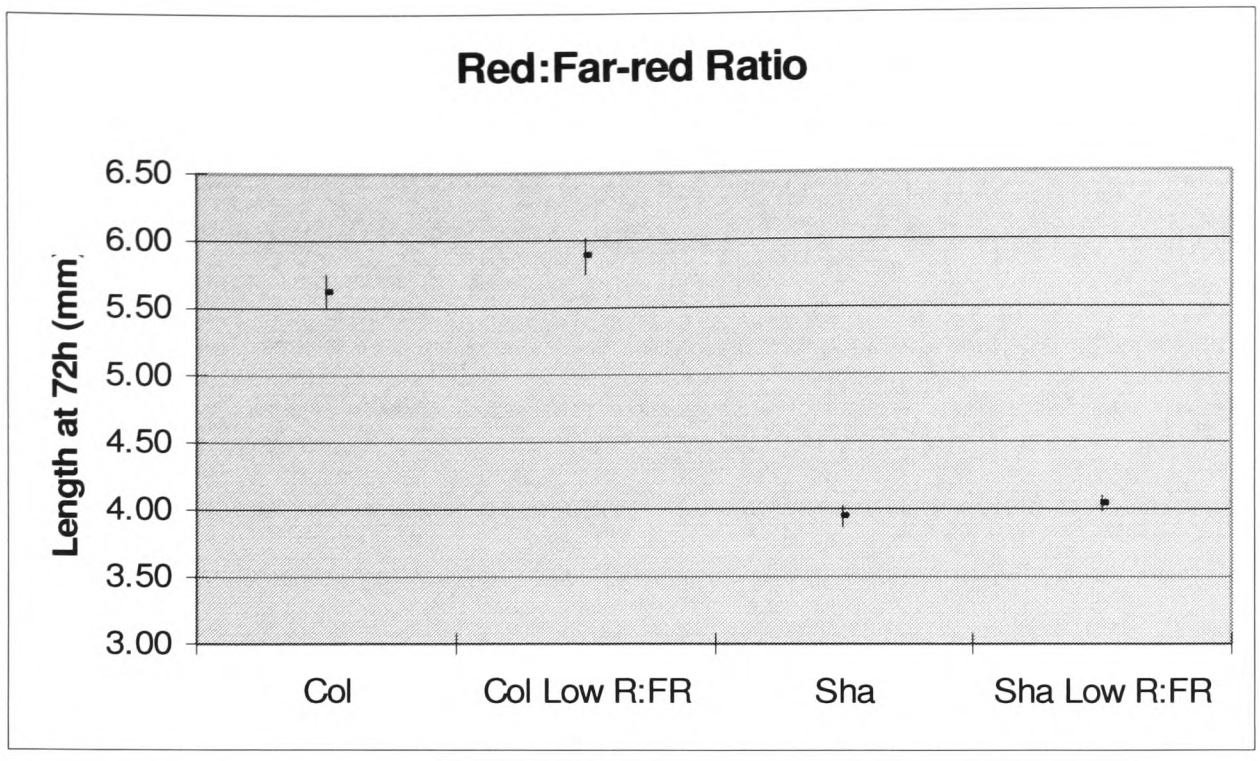


Figure 4.7. Effect of changing light quality on Columbia and Shahdara early root growth.
Mimicking competition by reducing the red:far-red light ratio does not have a significant effect on either genotype. Plant numbers: about 100 plants per genotype per treatment.

4.5.3 Light quantity effects on Columbia and Shahdara early root growth

Light quantity can also vary over the experimental area, due to plants being at different distances from the light source. To test for a light quantity-dependent effect on early root growth, Columbia and Shahdara seeds were germinated in the dark (by wrapping plates in foil to block out all light), under neutral shade (by providing a shielding of filter paper between the light source and the plates) and in full light. All other experimental conditions were maintained. Columbia seeds showed an increase in early root growth in response to increasing light intensity, whilst Shahdara roots showed no growth response to changes in light quantity (see Figure 4.8). Unpaired t-tests for root growth in dark versus full light conditions showed a highly significant difference for Columbia roots ($p = 5.92\ 10^{-21}$) but no significant difference for Shahdara roots ($p = 0.92$). Under dark germination conditions, Columbia and Shahdara roots grew to a similar length over the first 72 hours.

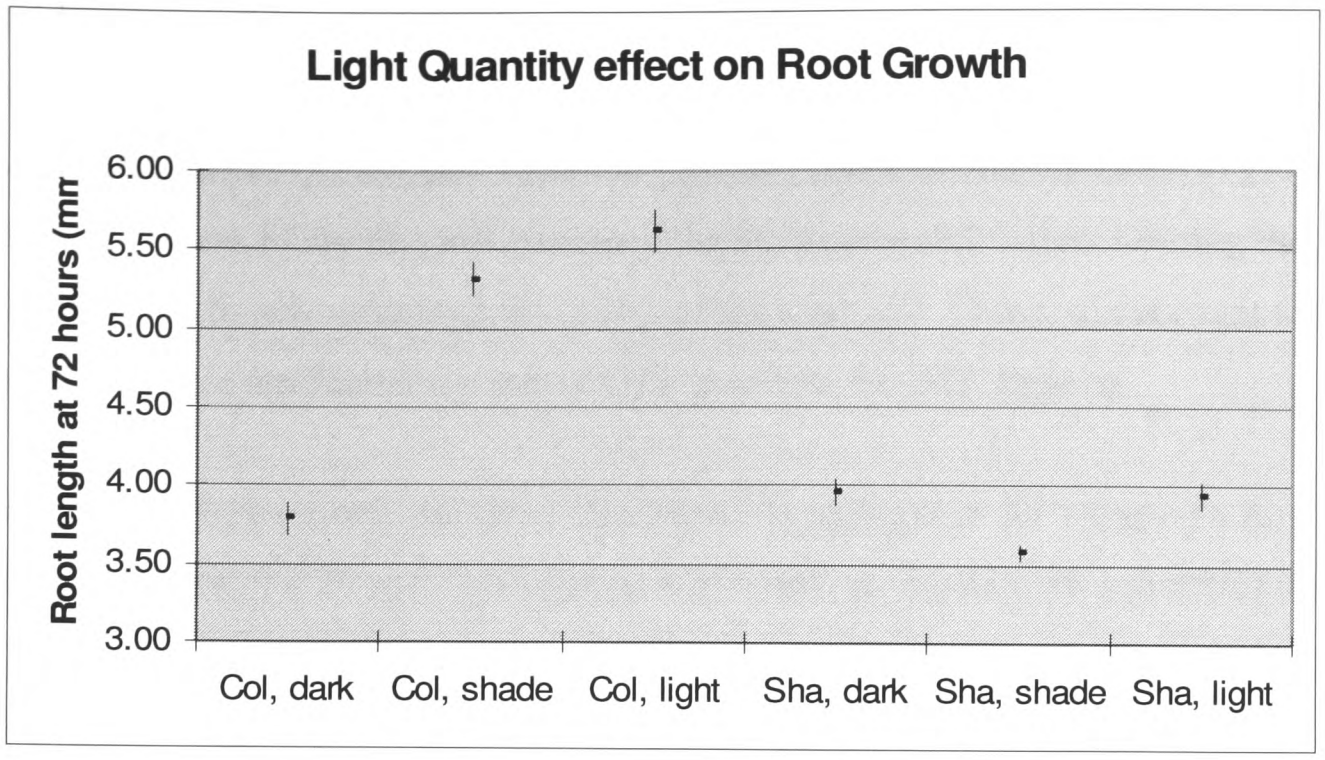


Figure 4.8. Light quantity effect on Columbia and Shahdara early root growth.
Showing that Columbia root growth increases with increasing light quantity, whilst Shahdara root growth shows no response to differences in light quantity in the first 72 hours of growth. Plant numbers: about 100 plants per genotype per treatment.

4.6 Discussion

Variation in root growth rate was observed in the Bay-0 x Shahdara RIL population. The mean root lengths of the RILs showed a distribution approximating to a normal distribution and extending in either direction from the mean root lengths of the parental lines. This spread of phenotypes – termed transgressive segregation – suggested that root growth rate was determined by multiple loci and that Bay-0 and Shahdara each carried a mixture of alleles, which promoted and reduced growth rate, so that the total genetic variance exceeded the difference in the parental means, thus making this population a good candidate for root growth QTL mapping. This choice of RIL population was also supported by the successful mapping of three primary root length QTL in a previous analysis of this population (Loudet, O. *et al.*, 2005).

A significant effect of environment on root growth was observed, due to nutrient composition and positional effects. The positional effects, observed during the QTL analysis, could have been due to differences in light intensity, temperature or

humidity across the experimental area and/or with time. Because the positional effects were not significant during initial experiments, which were carried out over a smaller area, the environmental variation probably reflected the area used to grow seedlings. It was likely that this increased the environmental variance during the QTL analysis, thereby reducing the power of the analysis. This could account for the low significance attributed to putative QTL positions by *QTL Express*.

Two QTL for root growth rate were identified – significant at the 1% level – on chromosome 4 (28 cM) and chromosome 5 (44 cM), explaining 7% and 8% of the variance, respectively. The amount of variance explained by these QTL was equivalent to the smallest of the QTL identified by Loudet *et al.* (2005). QTL of small effects are difficult to confirm or fine-map because they are easily masked by the effects of other loci or variation in the environment. A further two putative QTL were located on chromosomes 1 and 3, at 18 cM and 55 cM, respectively. Although their F-statistics were considered non-significant according to an experiment-wide permutation, this significance might have been reduced by the environmental interference in the experiment. Further reduction of environmental variance might therefore enable more accurate estimation of the location and effects of such QTL.

The QTL detected in this analysis can be considered novel QTL as none of them co-localised with those identified in the previous Bay x Sha primary root length analysis (Loudet, O. *et al.*, 2005). The lack of confirmation of previously identified QTL suggests a high dependence of root growth rate on environment, such that the differences between environments in the two experiments may have contributed to the differences in QTL detection. This would correlate with the hypothesis that root growth is very sensitive to environmental fluctuations - a trait which would improve the competitive ability of the plant if it could adapt early root growth to best suit its local conditions.

Attempts to confirm some of the putative root growth rate QTL identified in this analysis are described in Chapter 6 – Using Heterogeneous Inbred Families to Confirm QTL.

Early root growth was analysed in two parental genotypes with changes to three aspects of the environment to assess whether genetic-environmental interactions were apparent during early root growth. In normal conditions, at 72 hours after stratification, Shahdara roots were shorter than Columbia roots. This may have been due to a slower rate of root growth in the Shahdara line over this period, but was more likely a consequence of delayed germination of Shahdara seeds, which was observed in preliminary experiments. Neither genotype showed a significant response to changes in light quality, which suggests that roots do not respond to competition during this early stage of growth. Early root growth accelerated with increasing temperature, with both genotypes showing a similar gradient of response. Therefore, no genotype-specific effect of temperature was observed between these two parental lines; rather, temperature might intrinsically increase root length at 72 hours, either by promoting germination or by increasing the rate of root growth.

Changes in light quantity, however, affected Columbia, but not Shahdara roots significantly. Under dark conditions, Columbia root length at 72 hours was reduced to a similar length to Shahdara roots. As light intensity was increased, Columbia root lengths also increased, but Shahdara root lengths showed a slight decrease under neutral shade conditions and no significant difference between dark and full light conditions. It therefore appears that the environmental effect of light quantity was genotype-dependent, with the Columbia genotype interacting with the environment, but the Shahdara genotype being unresponsive. A possible explanation for this observed difference in light quantity response is that light quantity differences affect root growth only after some critical time-point or when roots have reached a certain length. As Columbia seeds tend to germinate earlier than Shahdara seeds, they reached this point during the course of the experiment and experienced an increase in root growth rate in response to increased light intensity. Meanwhile, Shahdara seeds tended to germinate later, and so this point was not reached during the experiment. An extended experiment would be required to assess whether, given more time, Shahdara root growth also responds to differences in light quantity.

From these simple experiments it is evident that different aspects of a plant's environment can affect growth significantly. Some of these variations are likely to

have a genotype-specific effect on growth, whilst others exhibit a general effect, causing all genotypes to respond in a similar manner. Either way, plants exhibit plasticity to their environment, which may be important for increasing fitness and competing successfully with surrounding plants. Variations in environmental conditions across the experimental area will therefore lead to differences in phenotypes by acting on this plasticity within plants. One effect of this will be to increase the environmental variance within RILs, thereby reducing the power of QTL analyses.

5 Rosette Relative Growth Rate

5.1 Introduction

A. thaliana rosettes expand by leaf initiation and growth. Leaves are initiated from rapidly dividing cells at the periphery of the shoot apical meristem (Fleming, A. J., 2005; Van Lijsebettens, M. & Clarke, J., 1998). After the first two true leaves, which are formed opposite each other, leaf primordia arise in a spiral pattern, each new primordium being formed at an angle of ~137.5 degrees from the last (Van Lijsebettens, M. & Clarke, J., 1998). Early leaf growth requires both cell division and cell expansion (Tsukaya, H., 2002), the regulations of which are thought to be related (Cookson, S. J. *et al.*, 2005; Fleming, A. J., 2002). This co-ordination has been seen to lead to an increase in leaf cell expansion to compensate for a lack of cell division in some leaf-development mutants (Tsukaya, H., 2003). For instance, in the *aintegumenta* mutant, leaves have fewer cells than wild-type, due to a reduction in cell division, but this is partially compensated for by an increase in cell expansion (Tsuge, T. *et al.*, 1996). However, the converse compensation does not seem to occur – over-expression of *AINTEGUMENTA* increases organ size by increasing cell number with no compensatory reduction in cell size (Tsuge, T. *et al.*, 1996).

Similarly, in the *angustifolia* mutant, a reduction in cell expansion is not compensated for by an increase in cell number, so leading to narrower leaves. *ANGUSTIFOLIA* (*AN*) is a carboxy-terminal binding protein that regulates expression of genes involved in cell wall loosening; mutants show an abnormal organisation of cortical microtubules that is responsible for a reduction in expansion of cells in the leaf-width direction (Kim, G. T. *et al.*, 2002). Rather than compensation by cell division for this reduced cell expansion, the reduction in cell expansion was seen to coincide with a reduction in cell division, resulting in fewer, thinner cells across the width of the leaf in *an4* mutants (Cookson, S. J. *et al.*, 2005). A similar effect on leaf blades was seen in the curly leaf mutant, *clf-25*, which had fewer, smaller cells in the leaf blade due to a reduction in rates of cell production and elongation (Kim, G. T. *et al.*, 1998).

Because leaf growth rates depend on the regulation of cell division and expansion, a QTL analysis of leaf relative growth rate (RGR) would be expected to identify factors involved either in intrinsic controls of division and expansion, or in signalling pathways by which rates of cell division and expansion are regulated. Because these measurements examine whole rosettes rather than single leaves, however, rosette RGR could also be affected by a difference in the rate of leaf production, so QTL analysis might detect modulators of the leaf initiation rate. Such modulations would produce a difference in leaf number at set time points.

A few QTL analyses of leaf rosette size and leaf number have been carried out in *A. thaliana*. Juenger *et al* (2005) measured the area of the third leaf in the Landsberg *erecta* x Cape Verdi Islands RIL population. These plants were grown under sterile conditions, on agar plates, under continuous light. Broadsense heritability for leaf area was calculated at 0.67, suggesting a strong genetic component, but only one QTL affecting leaf area was detected in this analysis. This was located at 85.7cM on chromosome 5, according to the L*er* x Cvi recombination map, and explained 13.4% of the total variance. Under the same conditions, but using the L*er* x Col RIL population, a total of 21 QTL were identified, affecting either juvenile (third) or adult (seventh) leaf and petiole traits (Perez-Perez, J. M. *et al.*, 2002). Perez-Perez *et al.* calculated broadsense heritabilities of 0.96, 0.87 and 0.93 for juvenile leaf area, adult leaf area and total leaf number, respectively. These particularly high heritability estimates they attributed to the stringent environmental controls in place during the experiment. Nine of the identified QTL affected adult leaf area with the percentage of variance explained by each varying from 3.9% to 14.6%. Two major-effect QTL were found on chromosomes 2 and 5 at 69.7 cM and 86.2 cM respectively. Although recombination maps cannot be directly compared between populations, a comparison of the markers used in the L*er* x Col and L*er* x Cvi maps (Alonso-Blanco, C. *et al.*, 1998) suggests that the QTL found on chromosome 5 in each study do not co-localise. This lack of correlation between studies is likely due to the different populations used. Juenger *et al.*'s lack of detection of the many small-effect QTL found in Perez-Perez *et al.*'s analysis may also reflect the lower heritability in the

former experiment. Increased environmental variance may have masked small-effect QTL, or there may be fewer genetic differences in the L*er* x Cvi RIL population.

Another QTL analysis in the L*er* x Col RIL population was carried out on plants grown in pots of compost in an unheated poly-tunnel in long days (16 hour light, 8 hour dark; Kearsey, M. J. *et al.*, 2003). Broadsense heritabilities in this experiment ranged from 20% to 40% for the various traits analysed: these low heritabilities probably reflect the reduced consistency of environment compared to the two analyses described above, which were carried out in very stringently controlled environments. However, Kearsey *et al.*'s conditions are closer to the natural growth environment of *A. thaliana*. Rosette size was analyzed at three time points – 21, 26 and 36 days after sowing – and two QTL were identified. One co-localised with the *erecta* mutation on chromosome 2; the other was located on chromosome 4 at 62cM, a position which was also identified by Perez-Perez *et al. (2002)* as having a small effect on juvenile leaf area. Interestingly, the *ERECTA* locus did not affect leaf area in the L*er* x Cvi analysis (Juenger, T. *et al.*, 2005) and affected the area of the third, but not the seventh, leaf in the previous L*er* x Col analysis (Perez-Perez, J. M. *et al.*, 2002). *ERECTA* encodes a Leucine-rich receptor-like serine/threonine kinase, which has been implicated in the control of organ growth by promotion of cell proliferation (Shpak, E. D. *et al.*, 2004). The low occurence of *ERECTA* detection in the above QTL analyses suggests that its effect is not intrinsic to growth rate of the leaf [*erecta* is identified as a leaf-shape mutation, rather than affecting leaf size (Bowman, J. L., 1993)].

These inconsistencies are indicative of the difficulties involved in QTL analyses. Even slight changes in the environment, measurement methods or population employed in the study can produce significantly different results. When QTL are located in more than one analysis therefore, their significance is greatly increased, implying a more general effect than environment- or population-specific QTL, which would be detected only under specific conditions. This further analysis of rosette RGR QTL adds another population to the analyses – the Bay x Sha RIL population – and assesses RGR behaviour in a different environment again to those in the studies mentioned above.

5.2 Variation for rosette RGR in the Bay-0 x Shahdara RIL population

Initially, two experiments were carried out to test for variation of rosette RGR in the Bay x Sha RIL population. The first experiment used 5 RILs (598, 600, 666, 677 and 777) plus the two parental genotypes; the second used 7 RILs (522, 600, 677, 698, 777, 823 and 881) and the parents. RGRs were obtained for each individual plant as described in Measuring Growth Rate (Section 3.1) and the means and standard deviations for RGR by genotype were calculated (see Figure 5.1). For each experiment, a one-way ANOVA indicated that there was some variation between genotypes (experiment 1: F-stat = 5.12, p-value = 0.00; experiment 2: F-stat = 2.66, p-value = 0.009). These two experiments could not be compared directly, as there was a general increase in RGR for all lines during the second experiment, which may not be linear, but RGRs were ranked in each case to compare those genotypes which were present in both experiments and thereby assess the reliability of RGR measurements. Rankings correlated well between experiments (ranked correlation coefficient = 0.9, $p < 0.05$; see Figure 5.2).

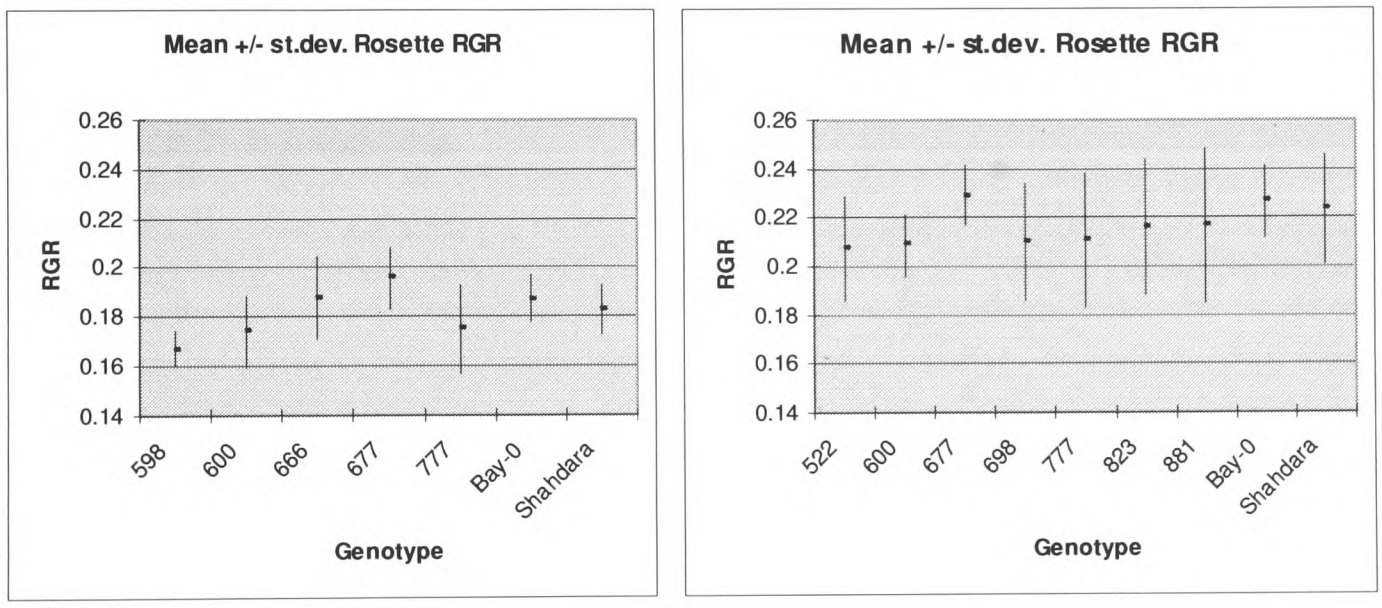


Figure 5.1. Distributions of RGR measurements for selected RILs of the Bay-0 x Shahdara RIL population.
Showing mean RGR extended either side by 1 standard deviation of the mean. First (left) and second (right) experiments were carried out under the same conditions but consecutively. 20 plants per genotype in each experiment.

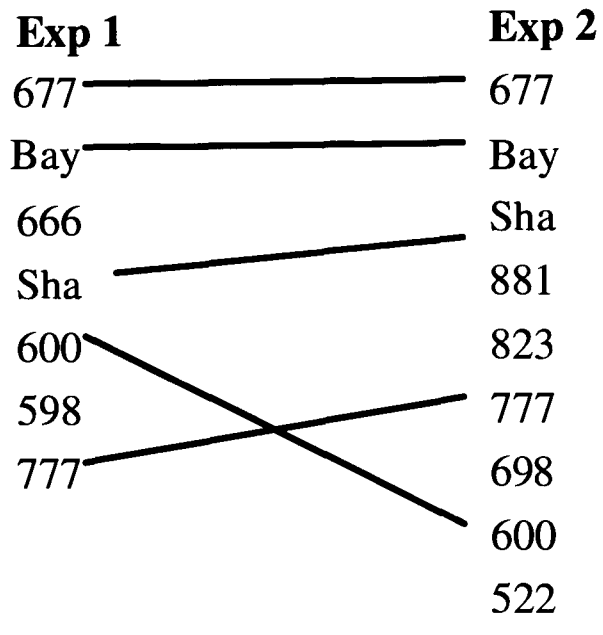


Figure 5.2. Comparison of ranked RGRs between experiments.
RILs and parents from the two initial RGR experiments ranked from fastest (top) to slowest growing, with connecting lines linking genotypes used in both experiments to show correlation in growth rate rankings.

Analysis of the rosette RGRs of the RILs used for the QTL analysis described below showed that there was variation for mean RIL RGR across the RIL population. RIL means tended to a normal distribution, with more variation apparent in the RIL population than was apparent in either of the parental lines (see Figure 5.3). Means for Bay-0 and Shahdara rosette RGRs in this experiment were 0.171 ± 0.002 and 0.146 ± 0.004, respectively.

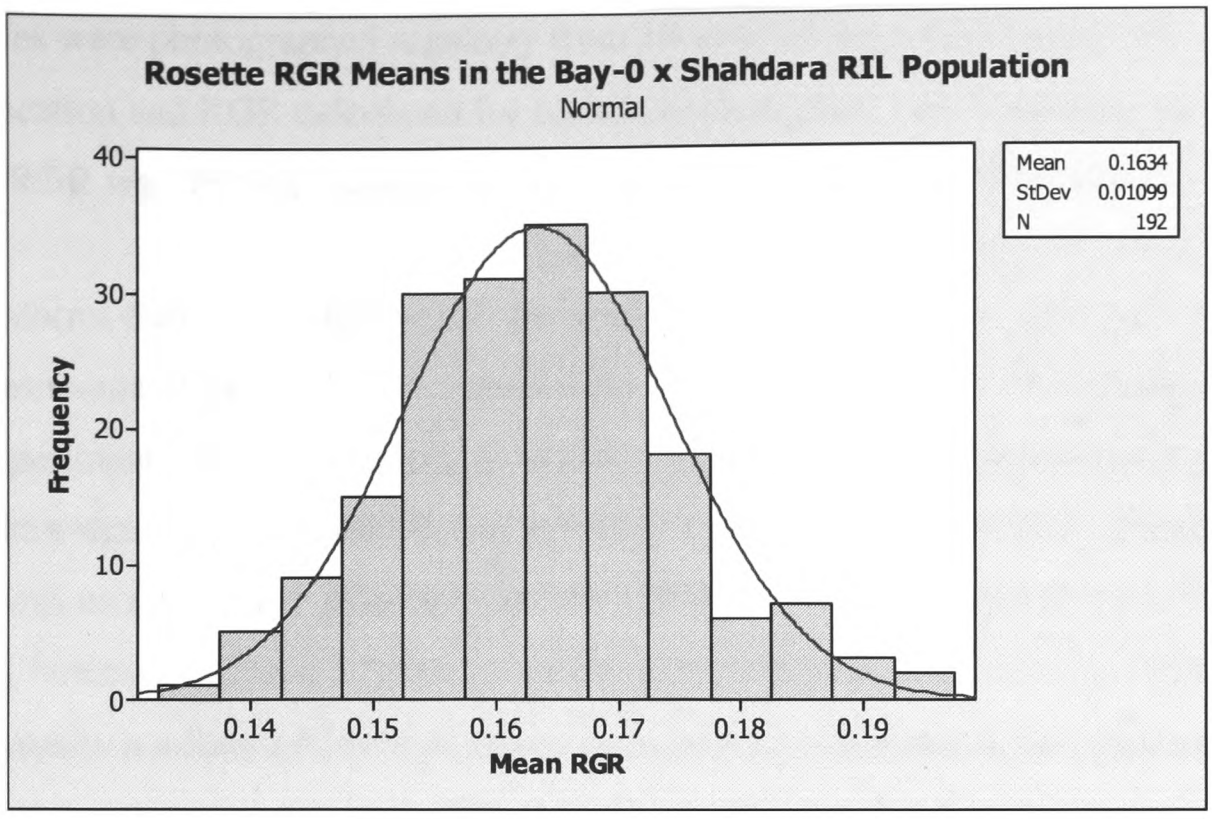


Figure 5.3. Histogram of RIL means of rosette RGR in the Bay-0 x Shahdara population.
Showing a normal distribution with mean of 0.163. Distribution of means of 192 genotypes.

5.3 QTL analysis of rosette RGR in the Bay-0 x Shahdara RIL population

The sub-set of 165 RILs and an additional 30 RILs were then grown for a QTL analysis of rosette RGR; 192 RILs were retained for the final analysis. Due to space and time constraints, the experiment was carried out in an overlapping block design. Ten replicates of about forty genotypes were sown in each block at intervals of one week between blocks. All seeds were stratified at 4° C for 72 hours, germinated on ½ MS medium (1% Agar, 0.6% Sucrose) and transplanted to soil after one week. All growth took place in the same growth room which was programmed to 20° C and short days (8 hours light; 16 hours dark). Plants were watered at regular intervals by standing the tray in water until the water was seen to have soaked up to the surface of the soil and then draining. Trays were shuffled and pots shuffled within trays frequently throughout the experiment.

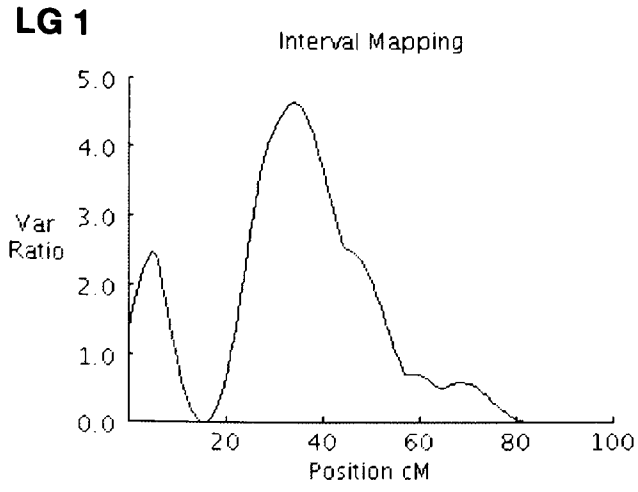
Rosettes were photographed regularly from 10 until 24 days after the end of stratification and RGR calculated for each individual plant (see Equation 3.1b). The mean RGR was then calculated for each genotype for use in the QTL analysis.

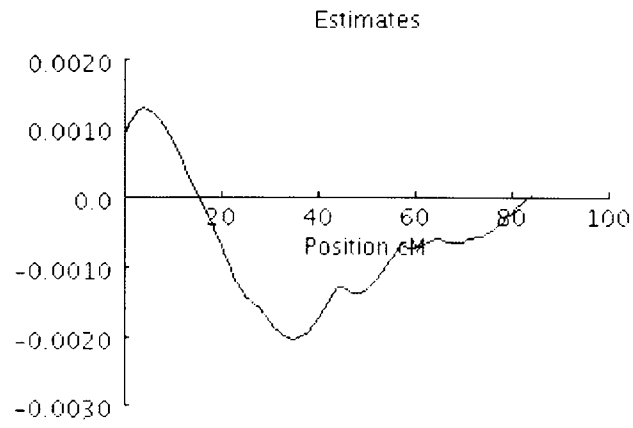
As a control during the experiment, parental plants were grown in each tray. The measurements of parental RGR values were used as a measure of the consistency of the experiment. Bay-0 and Shahdara RGR values were compared between trays. Shahdara showed little between-tray variation and a one-way ANOVA indicated that there was no significant difference between RGR values for this parent ($p = 0.997$). Bay-0, however, showed significant variation for RGR between trays ($p = 0.000$). Particularly, a reduced RGR was observed in tray 22 compared to the other values. This pattern was also apparent when the mean RGRs for all genotypes per tray were compared. Tray 22 was therefore considered an outlier and the data for plants in tray 22 removed in order to avoid this environmental effect affecting the QTL analysis.

Broad-sense heritability during this experiment was calculated as previously described (see equation 3.4), giving an estimated heritability of 43%. This was not as high as the estimated heritabilities observed in previous leaf growth QTL analyses (Juenger, T. *et al.*, 2005; Perez-Perez, J. M. *et al.*, 2002) but signified a reasonable level of genetic control for a quantitative trait.

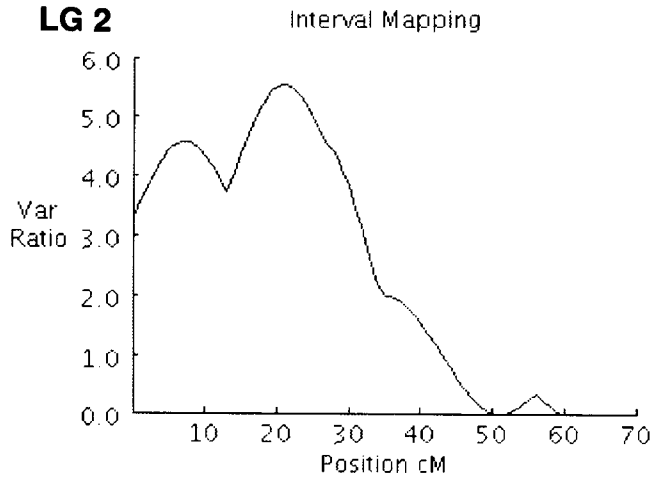
The QTL analysis was run in *QTL Express*, using mean RGRs for the 192 genotypes. An experiment-wide permutation test was used to set a significance threshold for the F-statistic of 11.13 ($p = 0.05$) or 13.77 ($p = 0.01$). Interval mapping identified only peaks below these significance levels (see Figure 5.4). The highest F-statistic, of 7.94, was returned at position 0 cM on chromosome 3. The estimated additive effect at this locus was 0.002, compared to a mean RGR of 0.165 ± 0.001 – i.e. the average difference between plants homozygous for the Bay-0 and Shahdara alleles at this locus was estimated to be 2.4% of the mean RGR.

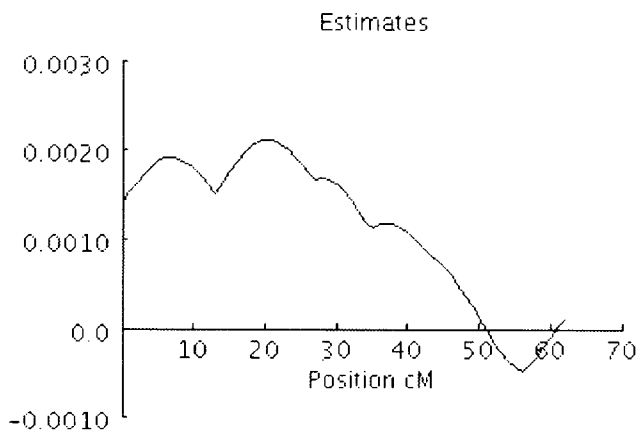
LG 1



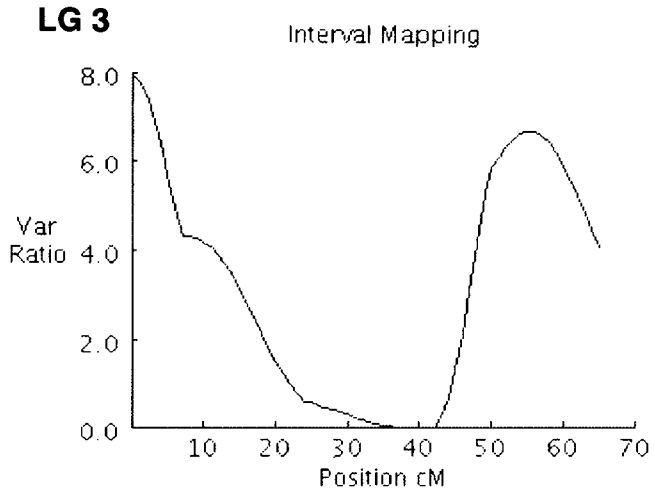


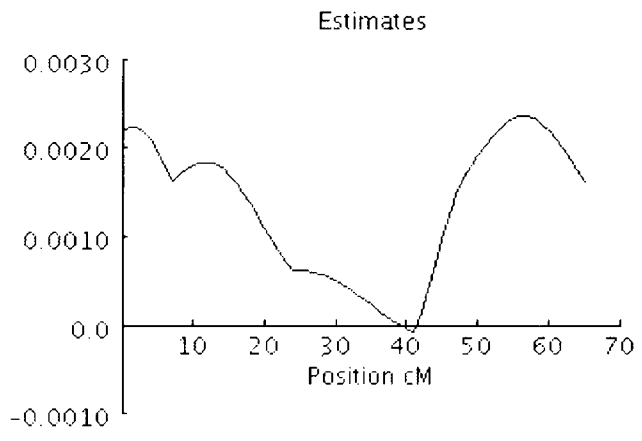
LG 2



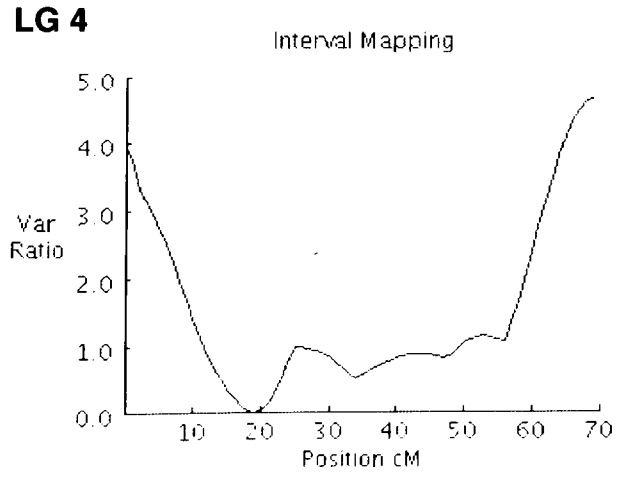


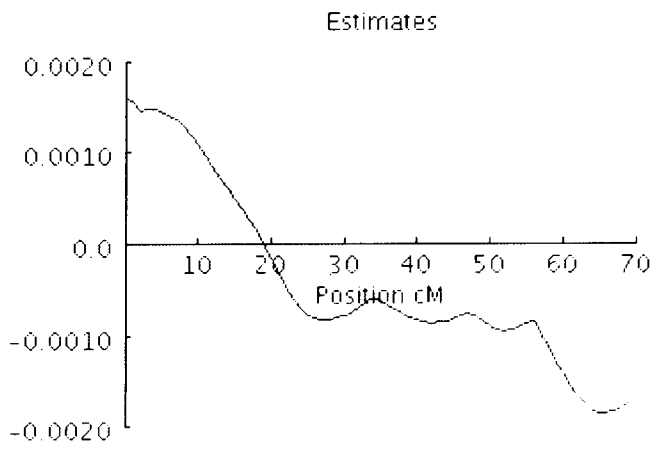
LG 3





LG 4





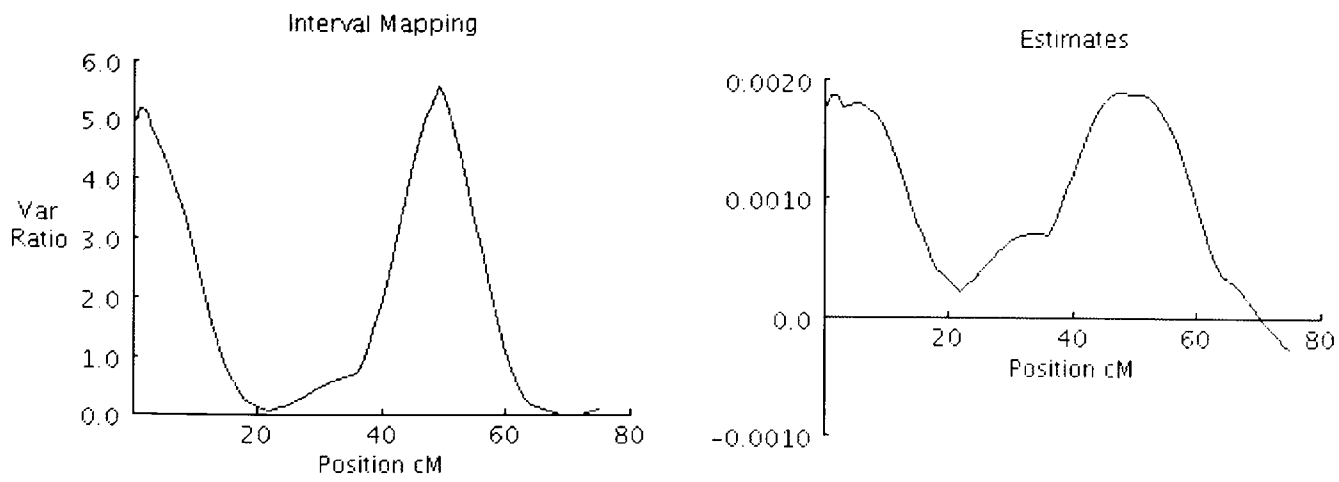


Figure 5.4. QTL analysis of rosette RGR in the Bay x Sha RIL population. *QTL Express* Interval Mapping output for rosette RGR, showing (left) variance ratio plots and (right) estimates of additive effects for the 5 linkage groups (LG1-LG5).

The best putative QTL for each linkage group are summarised in Table 5.1. Percentages of variance explained could not be calculated for these putative QTL because of the very small estimated additive effects.

Chromosome	Position (cM)	Additive Effect	F-statistic
1	34	-0.0020	4.65
2	21	0.0021	5.55
3	0	0.0022	7.94
4	69	-0.0018	4.68
5	49	0.0019	5.58

Table 5.1. Putative rosette RGR QTL.
Positions identified by *QTL Express* Interval Mapping analysis. Signs of additive effects are relative to the Bay-0 allele.

5.4 QTL analysis of leaf number

The rosette leaves visible 32 days after stratification were counted for each plant and a QTL analysis of mean leaf number per RIL was run in *QTL Express*. There was a significant difference in leaf number between Bay-0 and Shahdara genotypes, which produced an average of 9.5 and 7.3 leaves, respectively, over 32 days of growth (Student's t-test: p-value < 0.001). Although there was some between-block

variation for both parental lines, this was not significant at the 1% level (one-way ANOVA: $p = 0.024$ and $p = 0.029$ for Bay-0 and Shahdara respectively). A similar pattern was observed for leaf number variation in both parents, with the third block showing a particularly low leaf number compared to other blocks (see Figure 5.5), suggesting that there was an environmental effect that reduced leaf production in this block compared to the other blocks of the experiment.

The relationship between leaf number and RGR was assessed by plotting mean RGR against leaf number at 32 days for the 192 RILs used in the QTL analysis and calculating the Pearson's correlation coefficient (see Figure 5.6). This indicated that there was a low degree of positive correlation between the data. The coefficient of determination, r^2, was 0.144 – i.e. only 14% of the variation in RGR was explained by variation in leaf number.

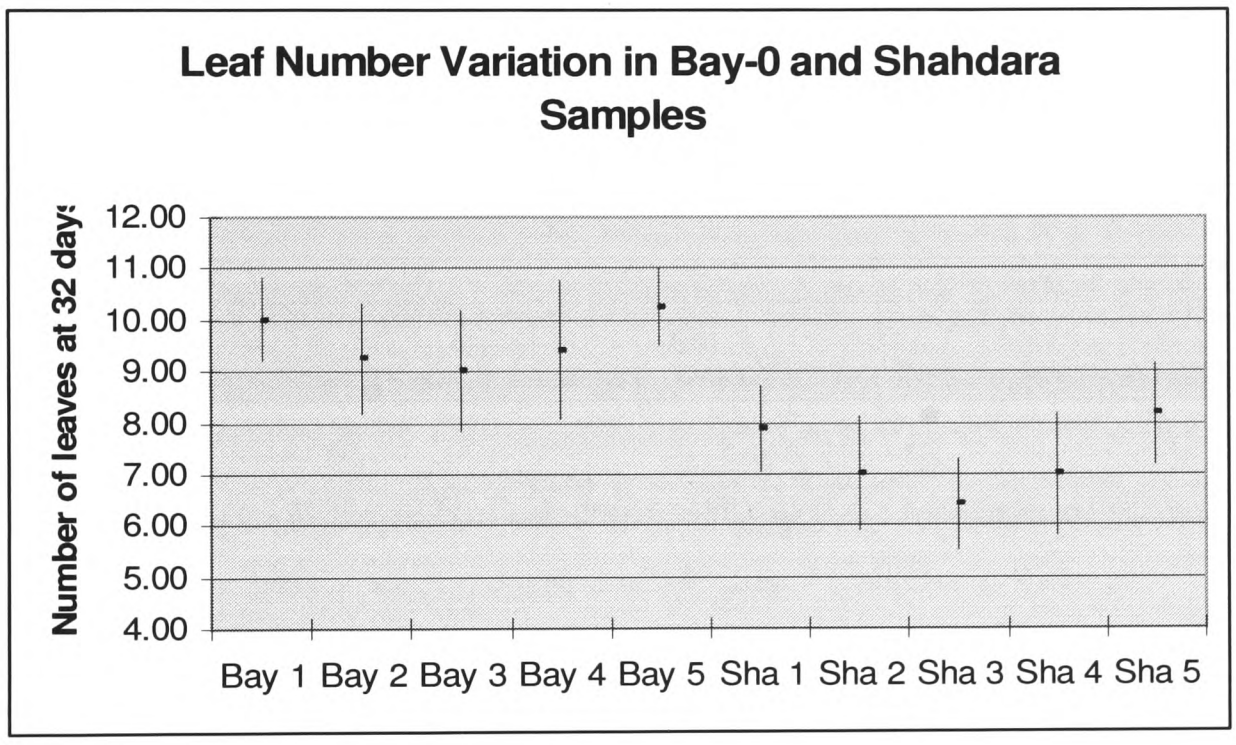


Figure 5.5. Leaf number variation in Bay-0 and Shahdara samples. Variation in the number of leaves 32 days after germination for parental genotypes arranged according to the blocks of the experiment, showing the mean leaf number with a spread of one standard deviation above and below for each block. Sample numbers: 10, 8, 24, 14, 11, 5, 10, 8, 12, 6, respectively.

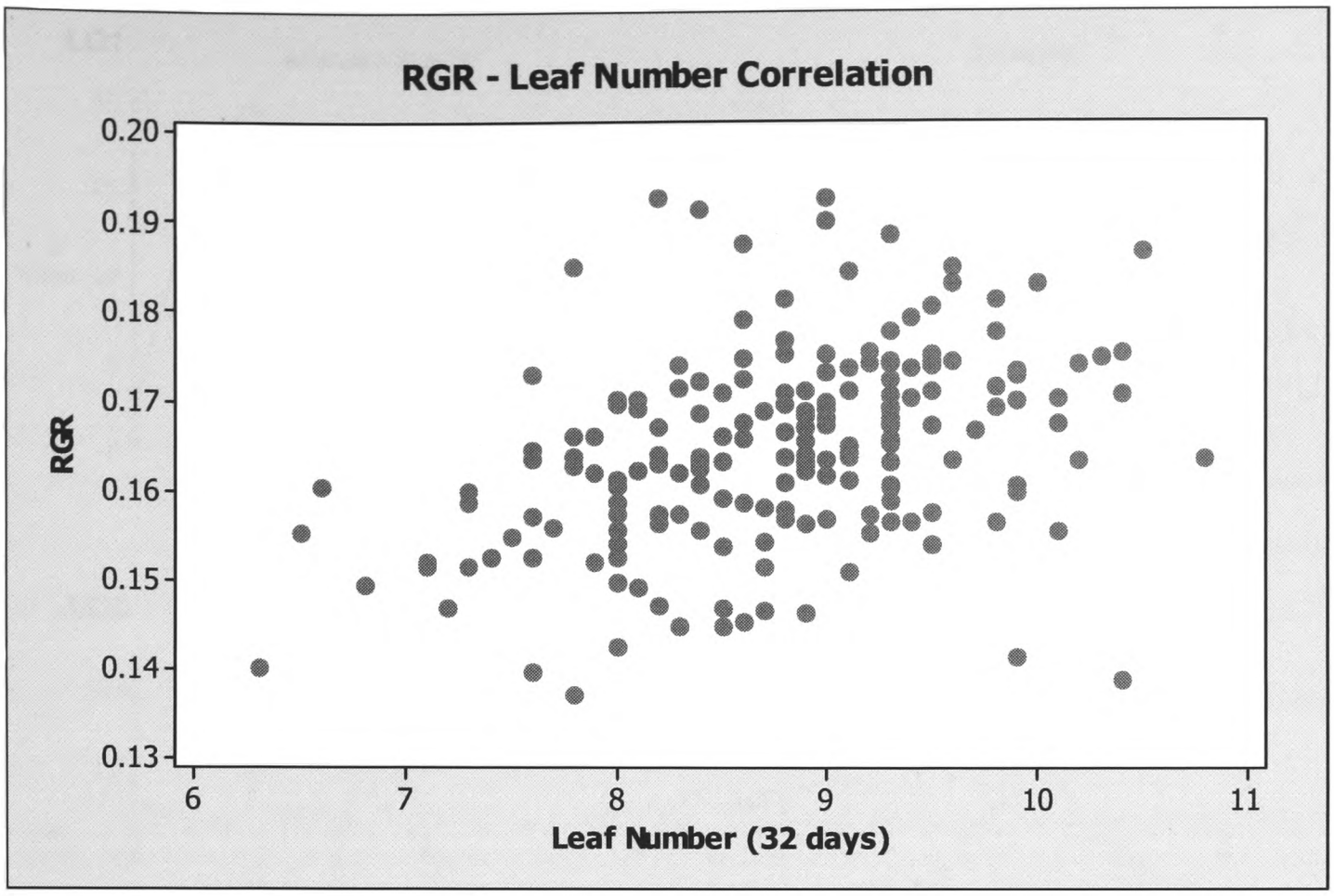
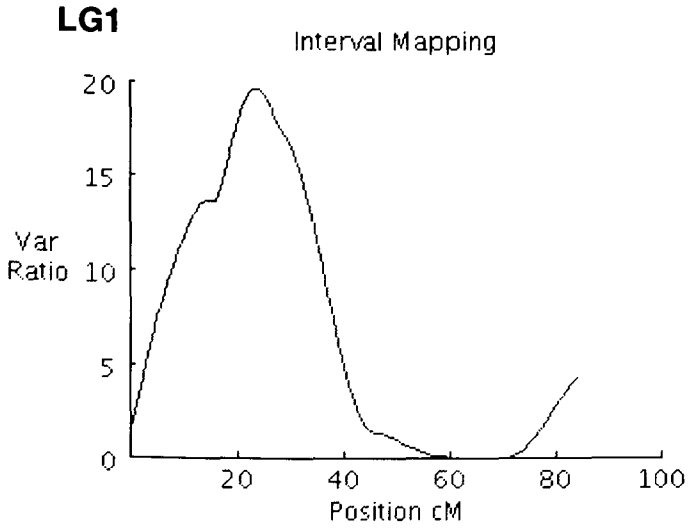


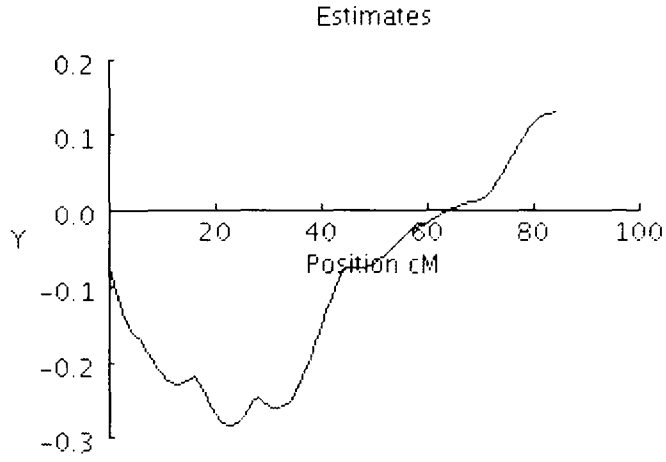
Figure 5.6. RGR – leaf number correlation.
Correlation between mean RGR and leaf number for 192 Bay-0 x Shahdara RILs; Pearson's product moment correlation, r = 0.379, indicating a low degree of positive correlation between the variables.

An experiment-wide permutation gave F-statistic thresholds of 10.6 or 13.6 for significance at the p = 0.05 or p = 0.01 level, respectively. Four significant QTL were identified in this analysis (see Figure 5.7). The positions, F-statistics, estimated additive effects (mean leaf number was 8.8 ± 0.06) and corresponding variance explained by each of these putative QTL (as described in equation 4.1) are summarised in Table 5.2.

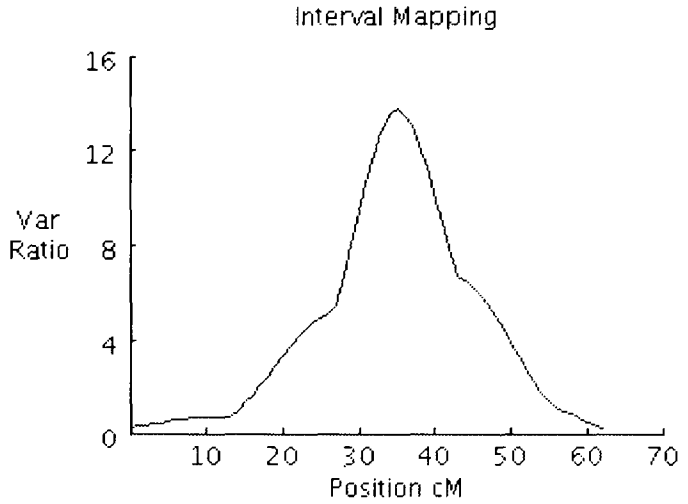
The variance ratio peak for chromosome 5 was dissected into two significant QTL using the 2-QTL search function in *QTL Express*. The effects of each of these two QTL were then analysed independently by setting each in turn as a background genotypic effect during interval mapping.

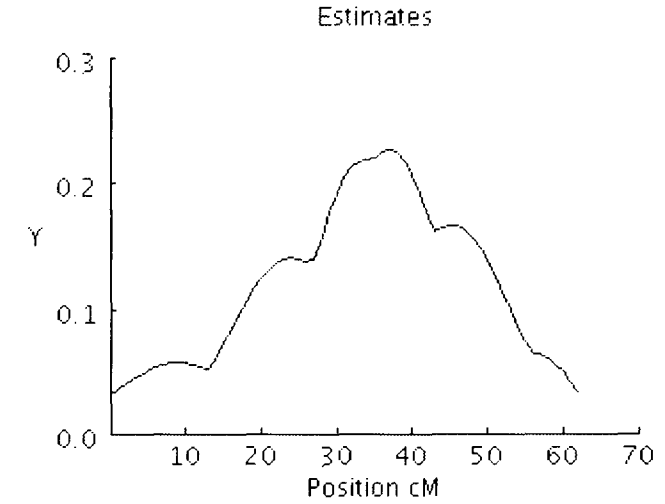
LG1


Interval Mapping
Var Ratio
20
15
10
5
0
20
40
60
80
100
Position cM

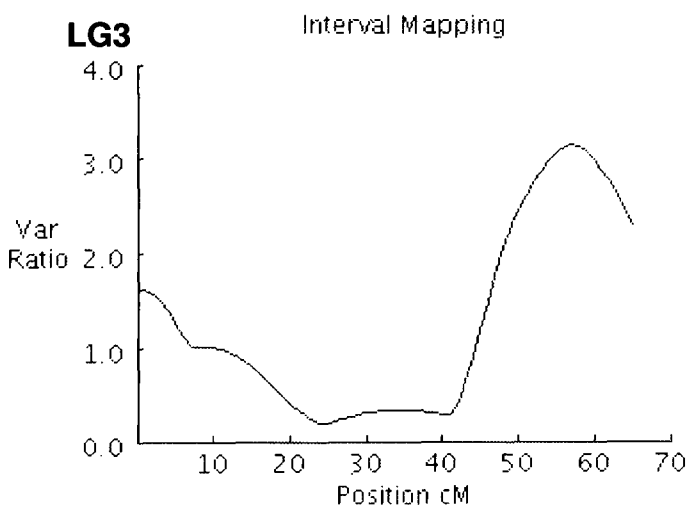

Estimates
Y
0.2
0.1
0.0
-0.1
-0.2
-0.3
20
40
60
80
100
Position cM

LG2


Interval Mapping
Var Ratio
16
12
8
4
0
10
20
30
40
50
60
70
Position cM


Estimates
Y
0.3
0.2
0.1
0.0
10
20
30
40
50
60
70
Position cM

LG3


Interval Mapping
Var Ratio
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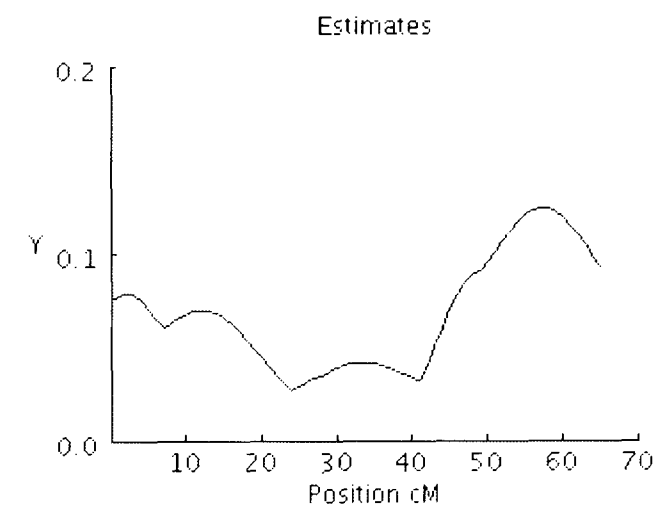
3.0
2.0
1.0
0.0
10
20
30
40
50
60
70
Position cM


Estimates
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10
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Position cM

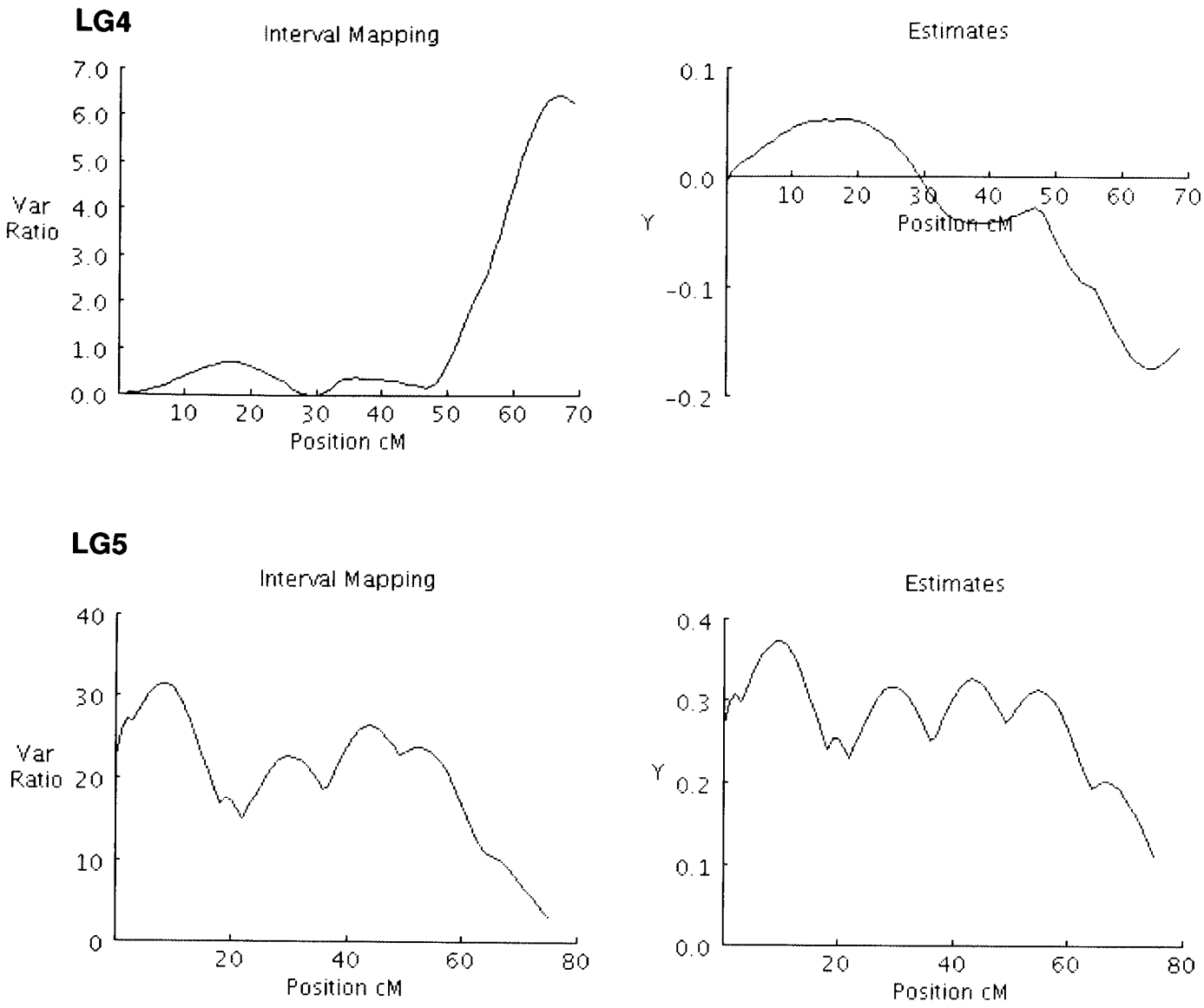


Figure 5.7. QTL analysis of leaf number in the Bay x Sha RIL population. *QTL Express* Interval Mapping output for leaf number, showing (left) variance ratios and (right) estimated additive effects for positions along each chromosome (LG1-LG5).

Chromosome	Position (cM)	F-statistic	Additive effect	Variance Explained (%)
1	23	19.68	-0.28	10
2	35	13.83	0.22	7
5	2	22.58	0.27	12
5	44	22.31	0.29	10

Table 5.2. Summary of putative leaf number QTL in the Bay x Sha RIL population.

5.5 Discussion

Initial experiments demonstrated the presence of genetic variation for RGR in the Bay x Sha RIL population. The estimated heritability for the trait was good, at 43%,

indicating that this was an appropriate population for QTL analysis. However the QTL analysis failed to identify any loci of significant effects. Generally candidate QTL were well below the significance threshold given by experiment-wide permutation and estimated additive effects of these loci were very small. The difficulties arising from the effects of environmental variance were evident in this experiment. This was reduced to some extent by removing from the analysis all data from tray 22, which showed abnormal values, but the environmental effect could not be completely removed and was too complex to be corrected for by data calibration. Possibly, genotype-environment interactions were also involved, giving differences in response to position between genotypes. For example, whilst Bay-0 RGRs varied considerably due to position, Shahdara plants seemed less susceptible to environmental changes, giving a non-significant ANOVA p-value when compared across trays.

One aspect by which Rosette RGR could be modulated is a change in the rate of leaf production, which would lead to a variation between plants in the number of leaves produced over a given period of time. Leaf number was analysed by counting the number of leaves visible by eye 32 days after stratification. Bay-0 produced on average 2.2 more leaves than did Shahdara plants over this period. There was some variation observed between blocks in the experiment, which was significant at the 5%, but not the 1% level. Unlike RGR during this experiment, leaf number variation for Bay-0 and Shahdara lines showed the same pattern of directional response across blocks of the experiment, with block 3 samples producing fewer leaves on average than the other blocks. This suggests conservation of the response of rate of leaf production to environmental changes in the two genotypes. A QTL analysis of mean leaf numbers identified 4 putative QTL, all significant at the 1% level according to an experiment-wide permutation. These QTL explained between 7% and 12% of the observed variance, three acting positively and one negatively in relation to the Bay-0 allele. There was also a variance ratio peak on chromosome 4 at 67 cM, although it was below significance with an F-statistic of 6.45.

The low correlation of leaf number with RGR implies that the leaf initiation rate does not substantially affect rosette RGR. Whilst 14% of the variation in rosette RGR was

explained by variation in leaf number in this analysis, the other 86% of variation must have been due to other factors – for instance the rate of cell division and expansion in leaf blades after initiation.

The greater ability of QTL analyses to detect leaf number rather than rosette RGR may indicate a higher degree of plasticity in RGR, indicating that growth of individual leaves is more adapted to the environment than overall plant growth by leaf initiation. Thus, environmental effects would influence RGR to a greater extent than leaf number, thereby adding phenotypic variation to the experiment and potentially masking small-effect QTL. Alternatively, this may be simply due to the size of QTL present in the population, with a smaller number of large-effect QTL affecting leaf production compared to many small-effect QTL contributing to natural variation in RGR.

Two putative rosette RGR QTL localised to similar positions as putative leaf number QTL – chromosome 4 at 69 cM (rosette RGR) and 65 cM (leaf number; below significance threshold) and chromosome 5 at 49 cM (rosette RGR) and 45 cM (leaf number). The additive effects of these putative QTL act in the same direction, to decrease RGR or leaf number on chromosome 4 and to increase RGR or leaf number on chromosome 5 in the Bay-0 allele. This co-localisation of effect strengthens the likelihood of QTL existing at these positions. There were no obvious correlations between the putative QTL identified here and those identified for earlier analyses of leaf area or number through indirect comparison of recombination maps. This, however, is not surprising due to the different population and growth conditions used in this study in comparison to those mentioned previously.

6 Using Heterogeneous Inbred Families to confirm QTL

6.1 Introduction

A heterogeneous inbred family (HIF) is a type of near inbred line derived from a RIL (Loudet, O. *et al.*, 2005). HIFs are a useful resource for the dissection of QTL effects, enabling the analysis of phenotypic differences caused by a change in the genotype of one chromosomal region between a pair of HIFs. The background genotype consists of a mixture of the two original parents of the RIL population, and is identical in a pair of HIFs. At the position of interest (the region around a putative QTL, for instance) the two HIFs differ for genotype, one parental genotype being inherited in one line and the other in its partner (see Figure 6.1).

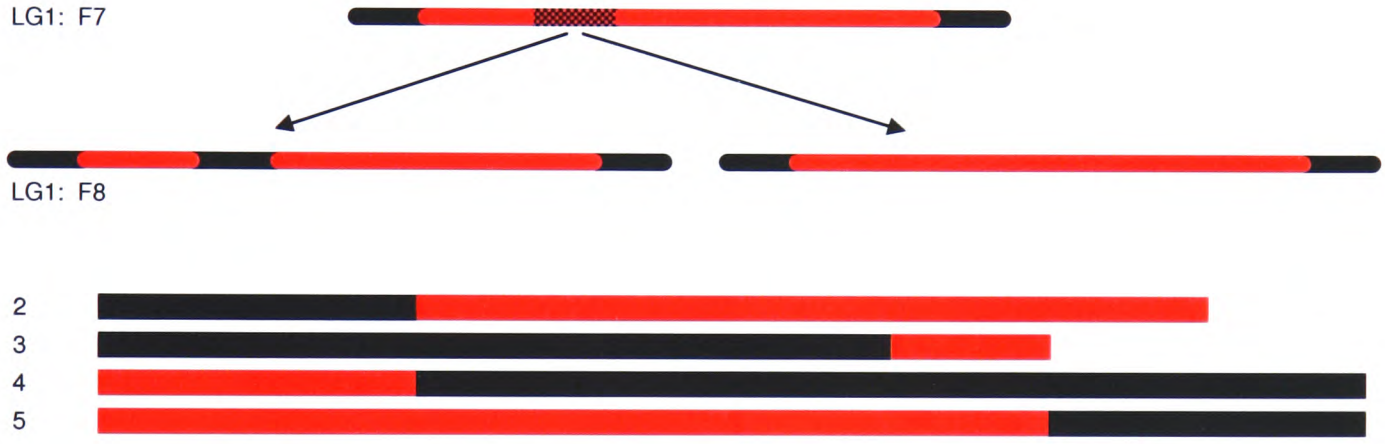


Figure 6.1. Schematic of the production of HIF 397 from the Bay-0 x Shahdara RIL 397.
Showing (above) chromosome 1 in the 7th RIL generation and the two (F8) HIFs and (below) chromosomes 2-5, which carry the same arrangements of parental genotypes in both HIFs. Black – homozygous for the Bay-0 allele; red – homozygous for the Shahdara allele; checked – heterozygous. Genotyping relates to markers used in analysis of the Bay-0 x Shahdara RIL population; not to scale.

HIFs are produced from the residual variation that is present in RILs. As the Bay x Sha RILs have reached only about 98% homozygosity at the sixth generation (Loudet, O. *et al.*, 2002), there is still a position in most RILs which remains heterozygous. RILs are therefore selected which show heterozygosity at the region of interest. These RILs are then self-fertilised and the progeny genotyped at the

region of interest, identifying the homozygotes for each genotype at this position. Pairs of lines are then used that share the common background of their parental RIL but carry opposing genotypes around the putative QTL (Loudet, O. *et al.*, 2005).

The theory behind HIFs and other NILs is that they allow the study of the effect of an individual QTL, if a small enough region around that QTL can be isolated in the NIL. This assumes that any other QTL are producing the same effect in both of the pair of HIFs, so does not account for any potential QTL-QTL interactions, which would interfere with the results. However, it does allow for the effect of one QTL to be verified and the position of this QTL further defined by means of introducing further breakpoints into the heterogeneous region for fine-mapping. HIF analysis also allows for preliminary analysis of QTL-environment interactions, by measuring and comparing the QTL effect in different conditions, before the exact position of the QTL has been identified.

Two sets of HIFs were used to verify the position of a QTL for primary root length originally mapped at 52.1 cM in the Bay x Sha RIL population (Loudet, O. *et al.*, 2005). By measuring root length differences between pairs of HIFs developed from RILs 90 and 196, which vary for two neighbouring regions of chromosome 4, Loudet *et al* confirmed the effect of this QTL, termed PRL3 (for primary root length 3) to within a 1.8 Mb interval on chromosome 4.

Use of HIF analysis for fine mapping QTL is advantageous because it reduces the sample number and simplifies the statistical comparisons required, by studying only two genotypes at one time, rather than the large numbers required for QTL analysis. As one of the major difficulties during the initial QTL analysis was maintaining constancy of environment over the necessarily large population numbers, it was hoped that QTL that registered with only low significance in the QTL analysis would become more apparent in their effects between HIFs, where there would be less environmental variability across the experiment. Also, some QTL effects might become magnified by slight alterations in conditions, such as nutrient modifications, which would be more easily adjusted and monitored over small sample numbers than during a full QTL anlaysis.

Therefore, several putative QTL positions were selected from the root growth rate and rosette RGR QTL analyses for further analysis in HIFs. The required HIFs were contributed by Olivier Loudet. The aim was primarily to verify the presence of QTL by increasing the significance of the effect. Further analysis would then include refining the confidence interval of the QTL and analysing the QTL effect in various environments.

6.2 Analysis of HIFs

6.2.1 HIF 90

HIF 90, originating from RIL 90 of the Bay x Sha RIL population, was received from Olivier Loudet and used to confirm the effect of PRL3, a QTL causing an increase in primary root length in the Shahdara allele (Loudet, O. *et al.*, 2005). HIF 90 segregates for the Bay-0 and Shahdara alleles at marker MSAT4.18 (positioned at 47.4 cM on chromosome 4) the nearest marker to position 52.1 cM, at which a QTL was mapped by Bay x Sha RIL analysis (Loudet, O. *et al.*, 2005). Loudet *et al.* (2005) previously observed segregation of a primary root length effect between the pairs of HIF 90. Seeds were sieved to remove early growth variation due to differences in seed size, sterilised and grown on vertical plates containing 0.5 × MS medium, 0.6% sucrose and 1% agar. Growth was measured from the third until the ninth day after stratification, quadratic regressions fitted to the data of root length against time and growth rates calculated for individual roots as described in section 3.2 - *Measurement of Root Growth Rate*. Comparisons of root growth rates at 3 and 9 days after stratification and the length of root extension during this time were made. Differences between Bay-0 and Shahdara alleles were marginally significant for day 9 growth rate (Two-sample t-test: $t = -2.08$, $p = 0.042$) and full length ($t = -2.09$, $p = 0.041$), with the HIF carrying the Shahdara allele showing a faster rate of growth.

The experiment was repeated with the concentration of sucrose in the medium increased to 2% whereupon the difference between Bay-0 and Shahdara alleles of HIF 90 root growth was enhanced. Mean root lengths for Bay-0 and Shahdara alleles were 31.85 mm and 36.79 mm, respectively ($\mu Bay - \mu Sha = -4.932$ mm). An

unpaired t-test indicated that this difference was statistically significant (t = - 6.26, $p < 0.001$). This effect corresponded to the estimated allelic effect of the QTL, PRL3, identified by Loudet *et al* (2005) in direction and magnitude.

6.2.2 HIFs for putative root length QTL

The HIFs measured for root growth rate and their corresponding predicted QTL from the Bay x Sha QTL analysis are shown in Table 6.1. Root growth was measured as for HIF 90, above, and pairs of HIFs were compared for evidence of QTL effects at segregating loci. Comparisons between pairs of HIFs 209, 397 and 194 for root growth rate at 3 and 9 days after stratification and for full root length showed no significant differences, indicating that there were no consistent QTL effects operating at these positions.

HIF	Segregating marker(s) (position)	Neighbouring markers (position)		Chromo-some	Putative QTL position (cM)	
		Upstream	Downstream		Root growth	Rosette RGR
194	MSAT1.10 (15.7 cM), NGA248 (26.9 cM)	F21M12 (5.7 cM)	T27K12 (43.6 cM)	1	18	23
397	NGA248 (26.9 cM)	MSAT1.10 (15.7 cM)	T27K12 (43.6 cM)	1	18	23
209	MSAT4.35 (24.8 cM), MSAT4.15 (34.1 cM)	NGA8 (16.4 cM)	MSAT4.18 (47.4 cM)	4	28	-
338	NGA172 (0.0cM), ATHCHIB2 (6.8 cM)	-	MSAT3.19 (23.9 cM)	3	-	0

Table 6.1. HIFs for putative QTL.
Showing respective segregating markers, their positions, non-segregating markers upstream and downstream, representing the maximum extent of the segregating region and the locations of putative QTL associated with these regions.

6.2.3 HIFs for putative rosette RGR QTL

Additionally, three HIFs that segregated for markers near putative rosette RGR QTL were measured for rosette RGR (see Table 6.1). Comparisons by unpaired t-tests between pairs of HIFs 194, 397 and 338 showed no significant differences for rosette RGR. Plants of several RILs with potentially mixed genotypes at markers linked to putative QTL positions were then genotyped for segregation at these loci and measured for rosette RGR. By this means, new HIFs were identified from RILs 128, 98 and 46 (segregating on chromosome 1 at marker F21M12) and 61 (segregating on chromosome 2 at marker MSAT2.36). However, there were no correlations again between HIF segregant genotypes and RGR according to unpaired t-tests on pairs of HIFs.

6.3 Discussion

In theory, using HIFs to confirm putative QTL for root and rosette growth rates is advantageous because of the reduction in sample number required, leading to a smaller experimental area and therefore reduced environmental variance. This should increase the power of the analysis. HIF analysis also allows independent investigation of a QTL, by studying the effects of changing genotype at one region only whilst maintaining a common genetic background. It should be noted that this does not take account of epistatic effects which may exist between the QTL and other loci; therefore a thorough investigation of QTL effect would require study of the QTL in several different HIFs in order to detect potential genotypic background effects.

This study was unable to confirm any putative QTL effects for root or rosette growth rates arising from the earlier QTL analyses. As many of the QTL were below the thresholds of statistical significance, the results of the HIF analysis were not surprising and merely confirmed the low likelihood of QTL at these positions. Additionally, the effectiveness of HIF analysis may have been reduced by the small effects of QTL, as suggested by the low additive effects and percentages of variance explained according to the QTL analyses. If QTL are present, but have only very small effects on the observed phenotypes, it may be very difficult to detect these in

the small populations used for HIF analysis. Furthermore, as nearly all putative QTL were studied only in one HIF background, it may be that QTL effects were masked in these genetic backgrounds by epistatic effects. A further explanation for the lack of QTL confirmation in HIFs would be the occurrence of recombination between the segregating marker and the QTL, such that the genotype of the QTL could not be predicted by the genotype of the marker. The probability of this increases as the distance between marker and QTL increases, so the closer the marker is to the putative QTL position, the stronger the power of HIF analysis. For this reason, it is also advantageous to study HIFs where pairs of markers segregate together, one each side of the putative QTL, as this reduces the likelihood of breaking the QTL-marker linkage by double-recombination events.

However, analysis of HIF 90 confirmed the presence of a primary root length QTL on chromosome 4, identified as PRL3 in a previous QTL analysis of the Bay x Sha RIL population (Loudet, O. *et al.*, 2005). The effect observed in the HIF was enhanced by increasing the concentration of sucrose in the medium from 0.6% to 2%, suggesting a limiting effect of the low sucrose concentration on root growth. At the higher sucrose concentration, the effect of the putative QTL was similar in magnitude to that previously observed by Loudet *et al*, with the Shahdara allele at the region of interest being associated with an increase in root length of about 5 mm at day 9. PRL3 is currently being fine-mapped by Olivier Loudet (personal communication) by means of identifying further markers within the confidence interval of this QTL, crossing the HIF partners and genotyping for new recombinants within this region. The recombinants will then be analysed for association between these new markers and the QTL effect in order to narrow the confidence interval of the QTL, thereby reducing the number of candidate genes within this interval.

7 Petal Growth

7.1 Introduction

At the completion of the vegetative growth phase, *A. thaliana* switches to its reproductive phase. This involves bolting – production of an inflorescence stem – and flowering. Flowers are small and consist of four whorls – sepals, petals, stamens and carpels. *A. thaliana* is a predominantly selfing plant. One study of insect visitors to *A. thaliana* flowers estimated an outcrossing rate of 0.84%, an estimate which is probably inflated as it assumes that every insect visitation results in an outcrossing event (Hoffmann, M. H. *et al.*, 2003). This very low rate of outcrossing correlates with the small size of *A. thaliana* flowers. Generally, plants which rely on insect pollination for fertilisation tend to have large flowers, often with colourful petals, to act as an attraction for pollinators. For example, the close relative of *A. thaliana*, *A. lyrata*, is self-incompatible and has correspondingly larger flowers than the inbreeding *A. thaliana* (Nasrallah, M. E. *et al.*, 2000).

Although occasional outcrossing events would be beneficial to avoid inbreeding depression by allowing opportunities for genetic mixing and adaptation, petals of *A. thaliana* appear to have very little function in terms of attracting pollinators. As anthers release pollen before reflexing of the petals (Weinig, C., 2002), petals may act to protect the internal parts of the developing flower, allowing pollination of the carpel to occur before the flower opens, exposing pollen to possible loss by wind. This would ensure fertilisation of the female gametes, whilst allowing the possibility of outcrossing by subsequent exposure of the pollen. Although this may explain the retention of the petal as a required organ, its size or growth rate is unlikely to be a major factor in this event.

Compared to leaves and roots, therefore, petal growth rate variation or adaptation to the local environment would have little effect on the fitness of the plant. As plasticity is likely to be a major contributing factor to the phenotypic variation observed in root and rosette growth rates, many of the QTL identified in such analyses may be involved in the environmental responses of these traits – i.e. they

will be environment-specific. If petal size were indeed less plastic, fewer genes for environmental response should exist, and therefore the majority of QTL identified in a QTL analysis would be involved in the intrinsic control of growth rate (i.e. cell division and elongation) and would appear across different environments. Additionally, experiments would be made easier by the reduction in environmental effect, as genetic variation would become the predominant source of variation in the trait. By this reasoning it was decided to carry out a QTL analysis on petal size in *A. thaliana*.

However, plasticity has been observed in *A. thaliana* petals in response to light quality, suggesting that petal development can be modified according to shading and competition (Weinig, C., 2002). While there seems little advantage for this adaptability, it is possible that, because of the close relationship between petals and leaves, genes involved in the intrinsic and environmental control of leaf growth may be expressed in petals by default. Hence the tendency of petals to revert to a leaf-like phenotype when floral identity genes are mutated (Pelaz, S. *et al.*, 2001). This would allow identification of general cell division and elongation rate QTL through analyses of petal growth, but also entails the likelihood of environmental variation affecting these measurements.

Variation for petal size has been observed amongst accessions of *A. thaliana* (Juenger, T. *et al.*, 2000) and QTL have been mapped in two RIL populations – L*er* x Col (Juenger, T. *et al.*, 2000) and Landsberg *erecta* x Cape Verdi Islands (Ler x Cvi; Juenger, T. *et al.*, 2005). Broadsense heritability for petal length was estimated at 0.58 for the L*er* x Col RIL population and thirteen putative QTL were identified as significant at the 5% level (Juenger, T. *et al.*, 2000). These putative QTL explained up to 30% of the total variance, the largest of which co-localised with the *ERECTA* locus on chromosome 2, with an estimated additive effect of 1.25 mm to a RIL population mean of 3.04 mm (the Col allele increased petal length). In the L*er* x Cvi RIL population, broadsense heritability was estimated at 0.67 and five putative QTL were identified for their effects on petal length (Juenger, T. *et al.*, 2005). Again, the *ERECTA* locus was identified in this study as having a positive effect on petal length, explaining 17.7% of the total variance with an estimated additive effect of 0.43 mm,

by the Cvi allele. No further direct comparisons can be made between these two populations because of the differences in recombination maps used to predict QTL positions. But, for example, a QTL explaining 10% of the total variance was localised to the bottom of chromosome 2 in the L*er* x Col RIL population, whilst nothing mapped to this region in the L*er* x Cvi RIL population. Similarly, a large effect QTL for petal length on chromosome 5 at 100 cM in the L*er* x Col map was not identified in the L*er* x Cvi population. These results suggest that different polymorphisms exist within the two RIL populations, resulting in detection of population-dependent QTL effects.

Eleven out of a total of eighteen floral morphology QTL mapped by Juenger *et al.* (2000) affected multiple floral traits, suggesting that these QTL were likely to function in the intrinsic control of cell division and elongation, rather than organ-specific growth (Juenger, T. *et al.*, 2000). This raises the possibility of these QTL also exerting an effect on leaf growth and possibly root growth. Three leaf morphology QTL were also mapped in the L*er* x Cvi RIL population, two of which co-localised with floral morphology QTL (Juenger, T. *et al.*, 2005). Other than that, however, there was only low correlation found between leaf and floral traits, suggesting that few co-regulatory factors acted during this experiment; six putative floral morphology QTL were identified that did not co-localise with leaf growth QTL.

It therefore remains to be determined whether the same genes affect growth rate of different organs of the plant – it is likely that a mixture of common and organ-specific controls exist. Comparisons between QTL analyses of growth in petals with those in rosettes and roots in the Bay x Sha RIL population may identify common growth controls, which would likely be indicative of general cell division and elongation controls. Additionally, QTL analysis of petal size in the L*er* x Col RIL population will allow direct correlation with the previous analysis by Juenger *et al.* (2000), although differences between environments may again affect the QTL detected.

7.2 Variation for petal size in two RIL populations

7.2.1 Variation in the Landsberg *erecta* x Columbia RIL population

Seventy-four RILs of the L*er* x Col RIL population, plus parental genotypes, were sterilised, stratified for 72 hours, sown on 0.5 × MS agar plates for germination and transplanted after one week into individual pots. Three individuals were grown per genotype and the plants experienced natural daylight in a glasshouse. Day-length was long as this experiment was run from mid-June to early July. Petals were collected and measured according to the method described in Materials and Methods Section 2.3 and mean petal area per genotype was calculated. An Anova of RIL petal area means showed that there was significant variation for mean petal area across this population ($F = 15.12$, $p = 0.001$). Mean petal areas varied from 1.1 mm^2 to 2.9 mm^2, with a mean of 1.8 $mm^2 \pm 0.03$ showing an approximately normal distribution (see Figure 7.1). RIL means spread outside of the Columbia and Landsberg *erecta* means, which were 1.5 $mm^2 \pm 0.04$ and 1.9 $mm^2 \pm 0.06$, respectively, suggesting that a number of antagonistic QTL contributed to petal size in the parents and were re-arranged in the RILs to show transgressive segregation.

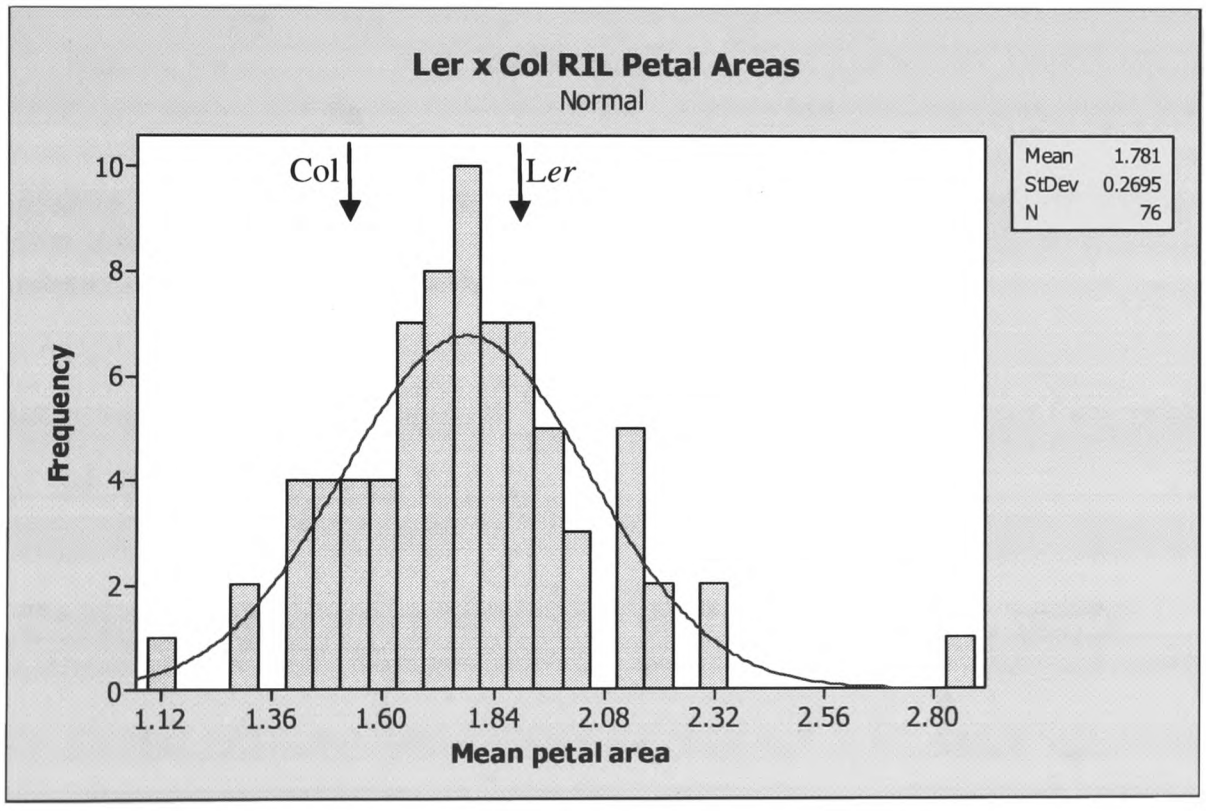


Figure 7.1. Distribution of mean petal areas for 75 genotypes of the L*er* x Col RIL population.
Arrows showing positions of parental means.

7.2.2 Variation in the Bay-0 x Shahdara RIL population

Variation for petal area was also apparent across 140 Bay x Sha RILs, which were grown in the same manner as above, but in short days (natural day-length from January to March). RIL mean petal areas showed a slightly skewed distribution around a mean of 2 mm^2 ± 0.05 (see Figure 7.2). This distribution was not 'normal' according to an Anderson-Darling test (AD = 1.863, $p < 0.005$). A wide variation in flowering time was also observed during this experiment, but has not been recorded.

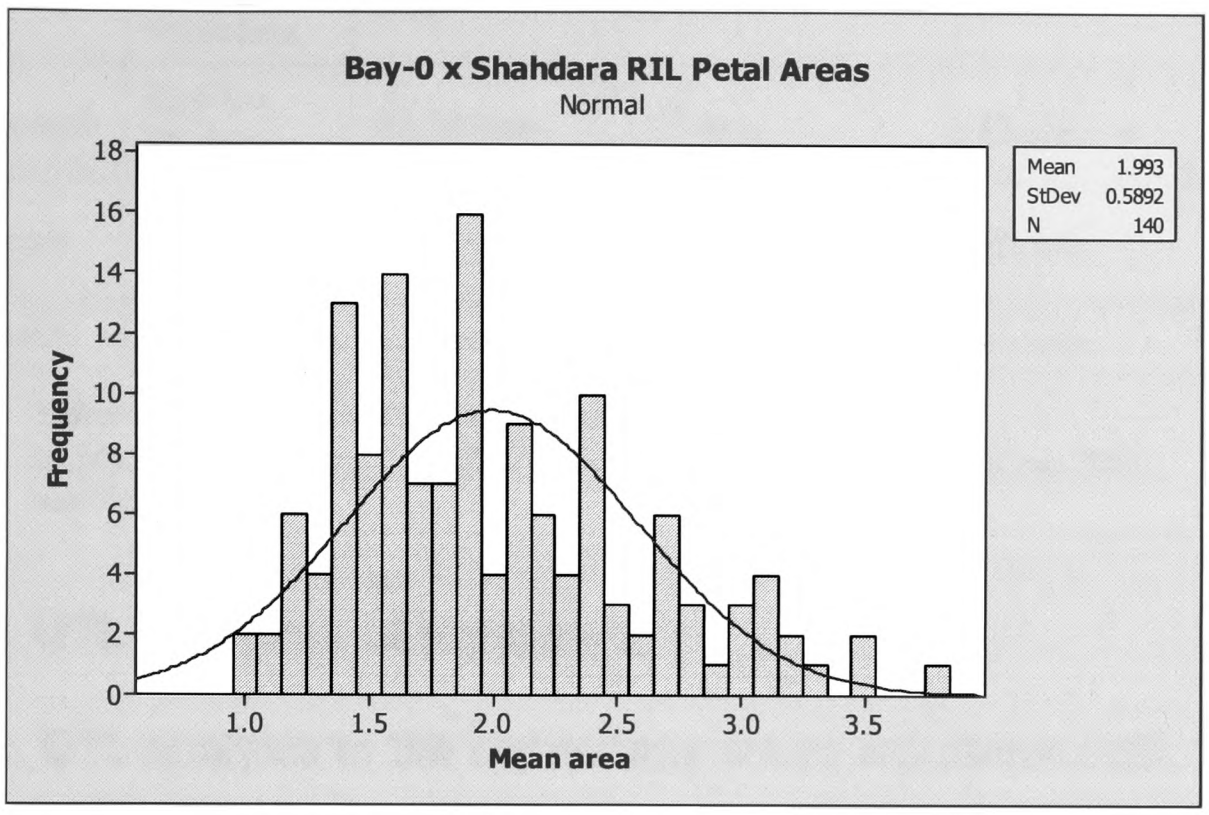


Figure 7.2. Distribution of mean petal areas for 140 genotypes of the Bay x Sha RIL population.
(Petal areas of parental lines have not been measured).

The natural variation for petal area in both of these RIL populations confirmed their potential for the identification of QTL.

7.3 Comparing environmental variance across traits

To assess whether environmental variance for petal size was smaller than those for root length and rosette RGR, the environmental variances in each of the QTL analyses were compared. Environmental variance refers to any variation which is not genetically determined; therefore it can be measured as the variation between genotypically-identical plants (within RIL variance). The average within-RIL

standard deviation was calculated as a percentage of the overall mean of the trait (see Table 7.1). Surprisingly, this showed a very low degree of environmental variance for rosette RGR compared to root length and petal area, which both showed comparatively high environmental contributions to variance.

Trait	RIL population	Overall mean	Average within-RIL standard deviation	Average standard deviation as percentage of mean
Rosette RGR	Bay-0 x Shahdara	0.16	0.01	6.4%
Root length	Bay-0 x Shahdara	43.24 mm	7.20 mm	16.7%
Petal area	Bay-0 x Shahdara	1.99 mm^2	0.32 mm^2	16.1%
Petal area	L*er* x Col	1.78 mm^2	0.22 mm^2	12.6%

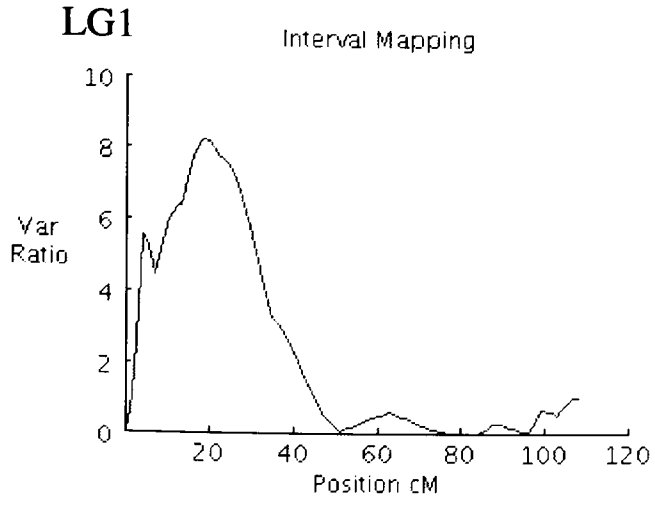
Table 7.1. Differences in environmental variance.
Estimated by standard deviation within RILs for three traits and two RIL populations.

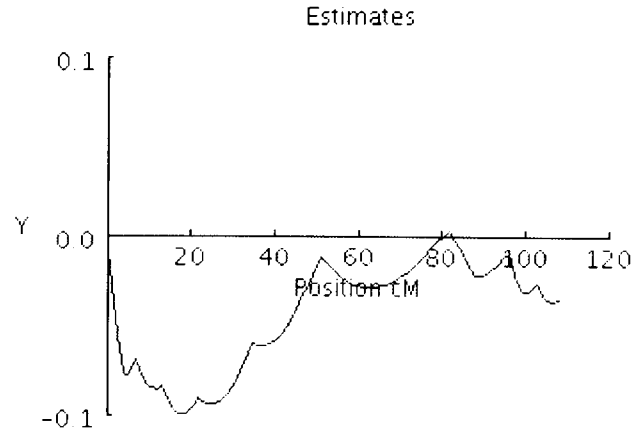
7.4 QTL analyses of petal size

7.4.1 QTL analysis in the Landsberg *erecta* x Columbia RIL population

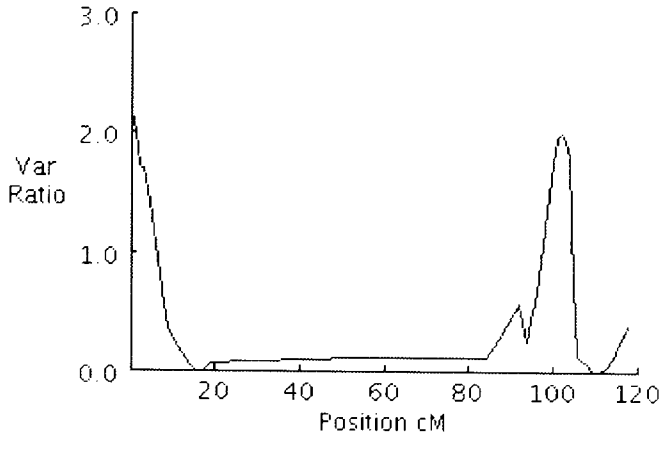
QTL analysis of mean petal area, using the L*er* x Col RIL population grown in natural long days, as described above, was run on *QTL Express* using 70 markers across the five *A. thaliana* chromosomes. An experiment-wide permutation indicated an F-statistic threshold of 11.2 or 14.7 for significance at the 0.05 or 0.01 probability level, respectively. By this restriction, there were no significant QTL identified in this experiment. The highest F-statistic returned from Interval Mapping in *QTL Express* was 8.26, for position 19 cM on chromosome 1. This locus had an estimated additive effect of -0.10 mm^2, explaining an estimated 10% of the variance. (See Figure 7.3.)

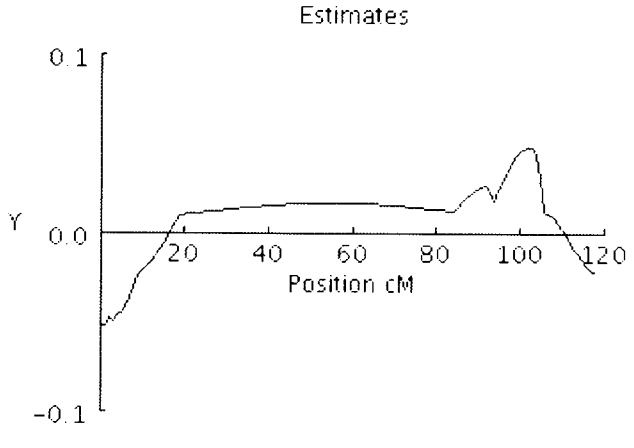
LG1



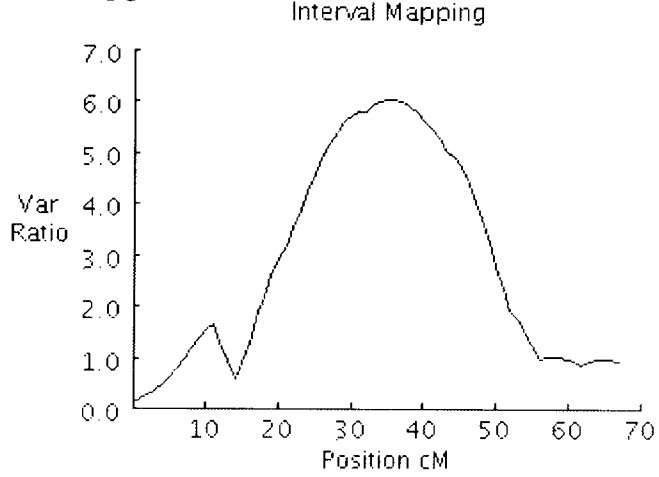


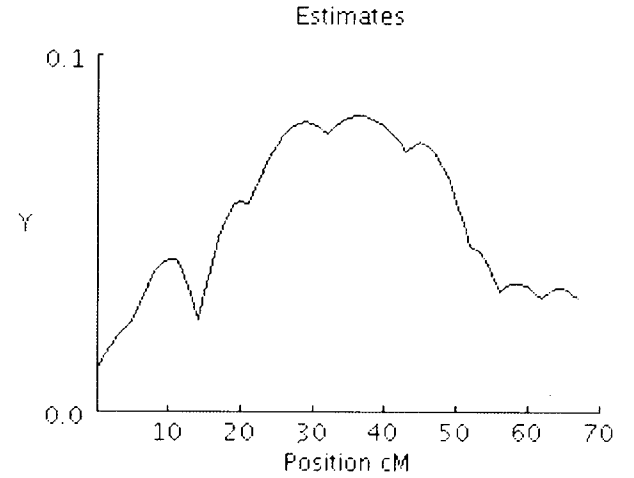
LG2



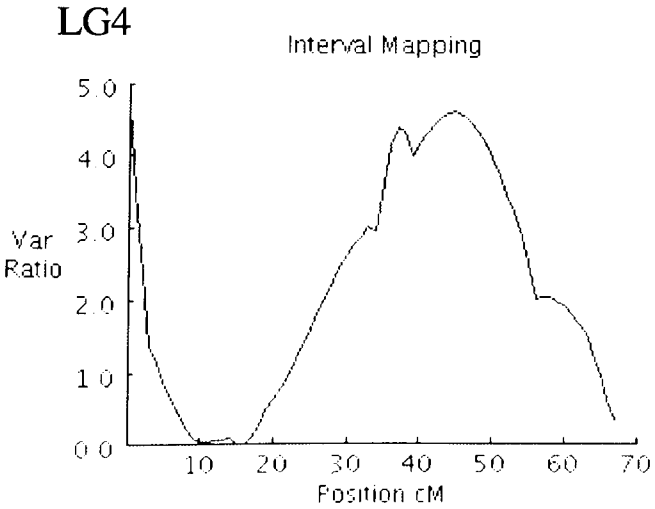


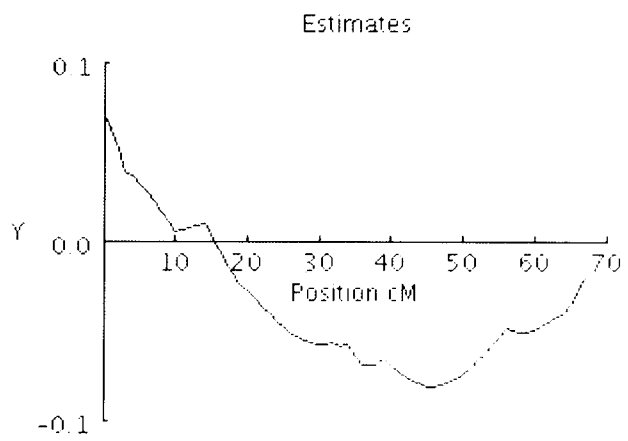
LG3





LG4





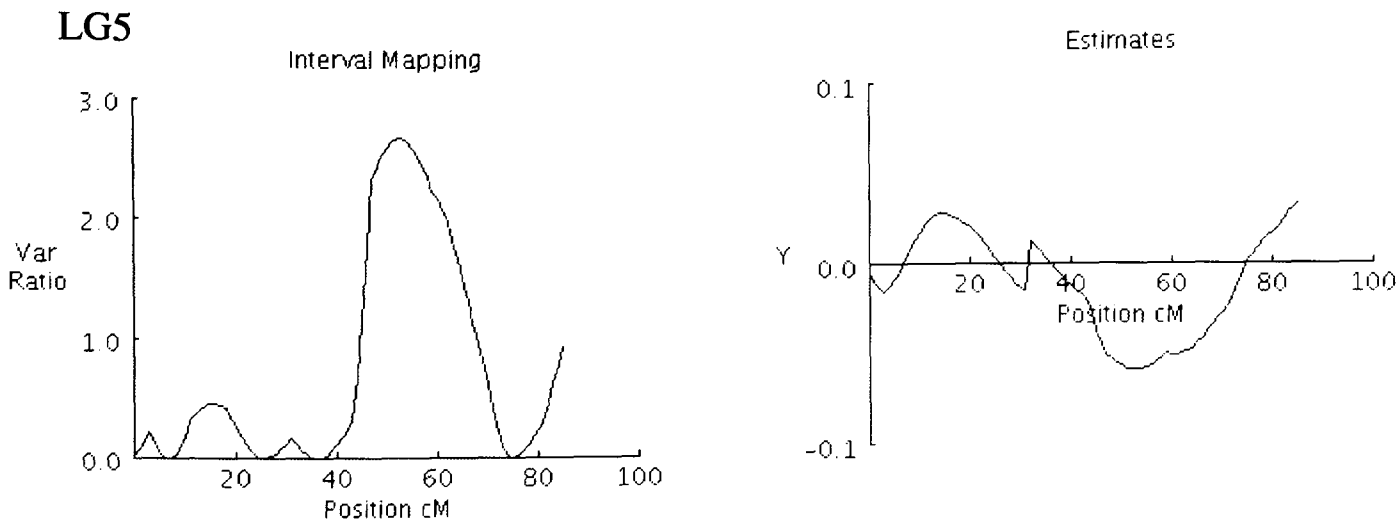


Figure 7.3. QTL analysis of petal area in the L*er* x Col RIL population. *QTL Express* Interval Mapping variance ratios (left) and estimated additive effects (right) for petal area, showing no QTL above the 0.5% significance level according to an experiment-wide permutation.

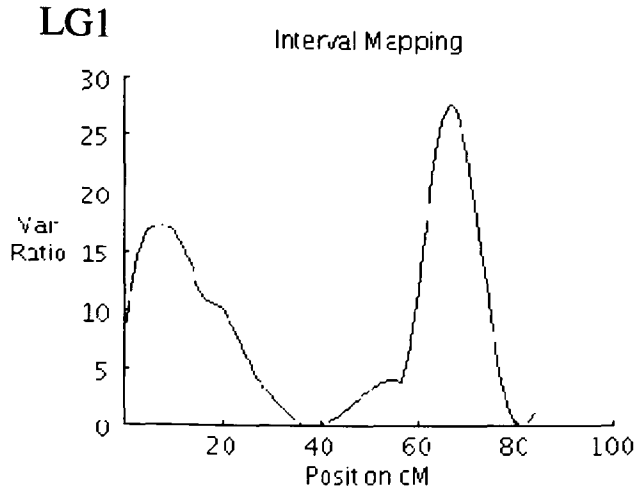
7.4.2 QTL analysis in the Bay-0 x Shahdara RIL population

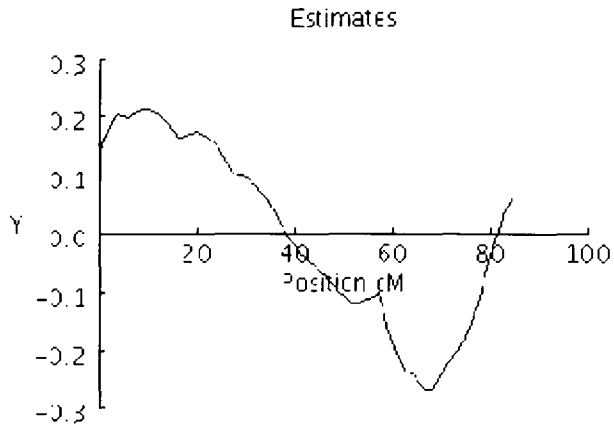
QTL analysis for petal area in the Bay x Sha RIL population by Interval Mapping in *QTL Express* identified three putative QTL that were significant at the 1% level according to an experiment-wide permutation test. (F-statistic thresholds of 10.6 or 14.4 were required for significance at the 0.05 or 0.01 probability level, respectively.) Two of these QTL were located on chromosome 1 and acted antagonistically. Their effects were dissected in *QTL Express* by entering each position as background genotypic effect. Positions were estimated at 10 cM and 67 cM, with additive effects of 0.21 mm and -0.27 mm, respectively. The third QTL was located on chromosome 4 at 0 cM, according to the Bay x Sha recombination map (see Figure 7.4; Table 7.2).

Chromosome	Position (cM)	F-statistic	Estimated additive effect (mm^2)	Variance explained (%)
1	10	20.98	0.21	15
1	67	27.78	-0.27	16
4	0	18.60	-0.23	15

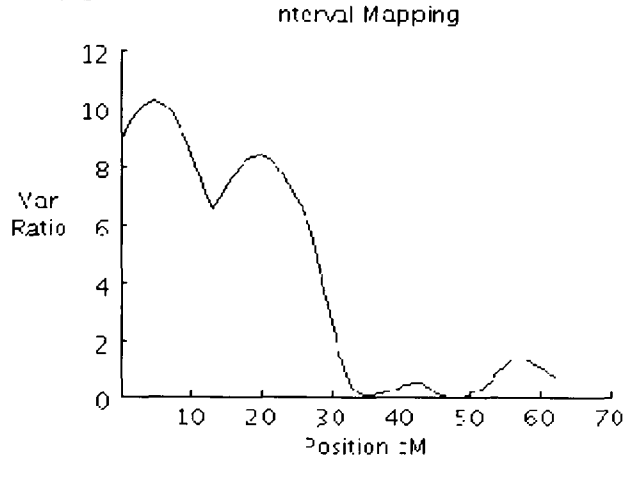
Table 7.2. Petal area QTL.
Summary of significant ($p < 0.01$) putative QTL positions and effects for petal area in the Bay x Sha RIL population. Positive estimated additive effects indicate an increase in area corresponding to the Bay-0 allele.

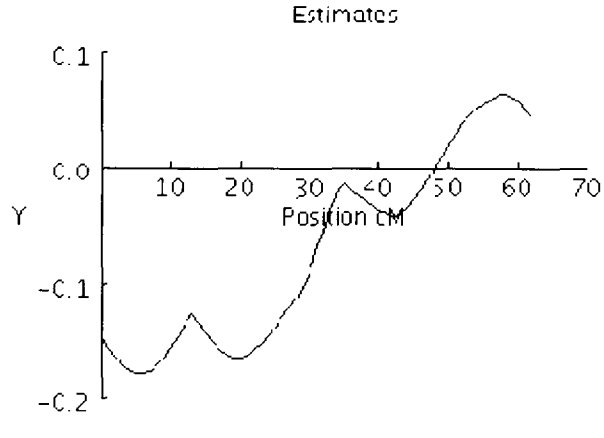
LG1



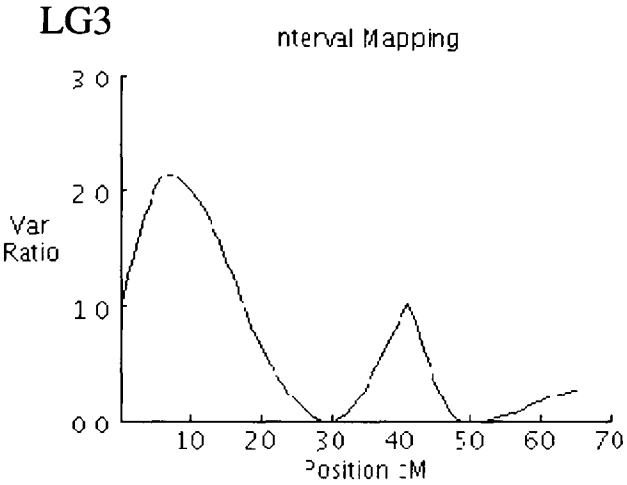


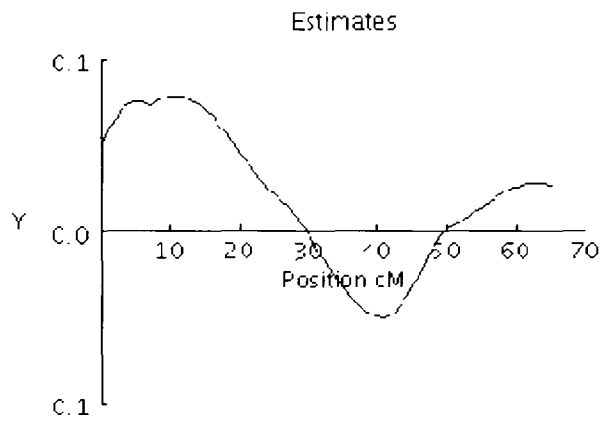
LG2



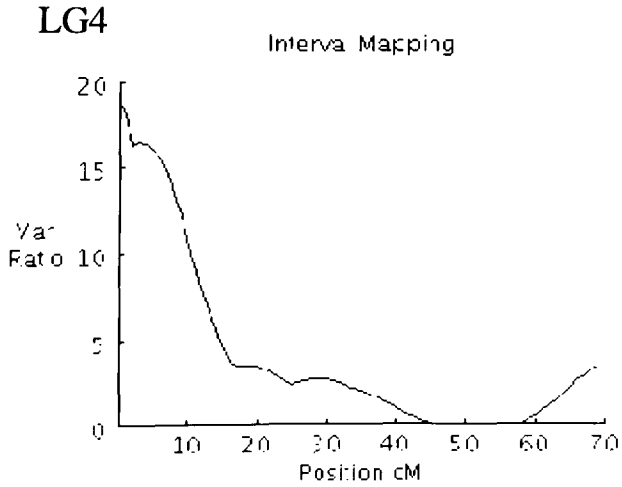


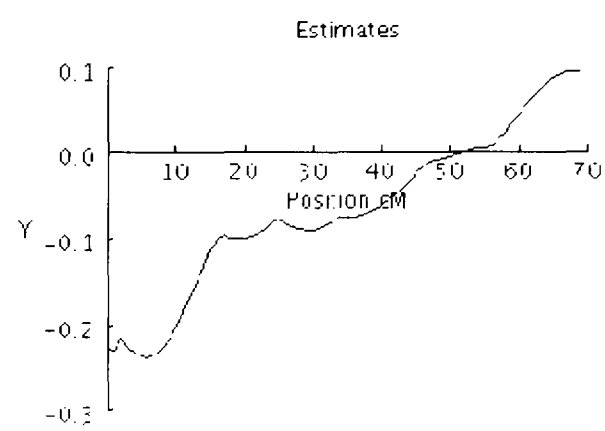
LG3





LG4





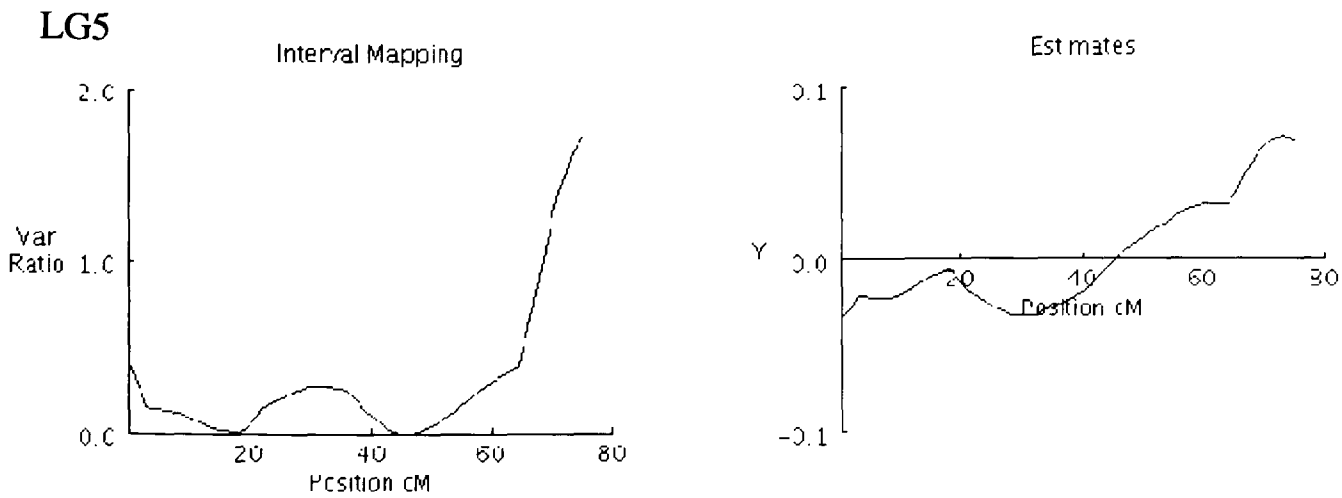


Figure 7.4. QTL analysis of petal area in the Bay x Sha RIL population. *QTL Express* Interval Mapping variance ratios (left) and estimated additive effects (right).

7.4 Discussion

QTL analyses for petal size have been carried out in two RIL populations under different environmental conditions. Transgressive segregation appeared in the L*er* x Col RIL population, which was grown under natural long-day conditions. However, no significant QTL were detected during this experiment. As QTL effects on floral morphology had been previously identified in this population, including the *ERECTA* locus, which explained an estimated 30% of the total variance (Juenger, T. *et al.*, 2000), it is likely that differences in environment have masked QTL in this experiment. Whilst both studies were carried out in long-day conditions, Juenger *et al.* (2000) used a controlled study area with 16 hours of light at 20° C and 8 hours of dark at 18° C. In contrast, this analysis was carried out in a glasshouse under natural day length of about 18 hours light, 6 hours dark with temperature (although partially controlled) varying considerably according to sunlight intensity, frequently rising above 30° C. These conditions caused plants to grow and flower very quickly, and possibly caused stress in the plants, masking the effects of QTL previously observed in this population.

The Bay x Sha RIL population was also grown in a semi-controlled glasshouse environment. Day lengths varied from 7 hours light, 17 hours dark at the beginning of the experiment to 11 hours light, 13 hours dark during flowering. Parental petal sizes were not measured for this population, so it cannot be ascertained whether

transgressive segregation occurred. However, the spread of mean RIL petal sizes suggested the presence of several QTL. This variation showed a slightly skewed distribution, with an increased number of RILs with small mean petal sizes than would be expected in a normal distribution of means. This may reflect a general environmental influence on petal growth of short day lengths causing a tendency towards reduced growth rates, so that flowers often reach maturity and open before petals have fully expanded. As QTL analysis programmes are mostly designed to work with normally distributed data, this may have affected the outcome of the analysis.

Three putative QTL were identified from the Bay x Sha analysis: two were localised to chromosome 1, explaining 15% and 16% of the total variance at 10 cM and 67 cM, respectively; the third QTL was mapped to chromosome 4 at 0 cM and explained an estimated 15% of the total variance. The two QTL on chromosome 1 acted antagonistically, petal area being increased by the Bay-0 allele at 10 cM and decreased by the Bay-0 allele at 67 cM. The similar magnitudes of these antagonistic QTL effects suggest that they would be cancelled out in parental strains. As the third putative QTL acts to decrease petal area in the Bay-0 allele, it would be predicted that Shahdara plants would have a larger mean petal area than Bay-0 plants under these conditions.

It was predicted that petals would be less adaptive to local environments than roots and rosettes and would therefore be stronger candidates for QTL analysis, allowing detection of intrinsic growth controls. This prediction was based on the seemingly low requirement for adaptation to local environments of the petal for plant fitness, as well as the short growth time of a petal in comparison to the growth of a leaf or primary root, exposing it to less potential fluctuations of the environment. Also, leaf morphology is obviously altered depending on the environment (Cipollini, D., 2005), whereas this is not seen in petals. However, the expected reduction in environmental variance was not observed. In contrast, rosette RGR appeared to have a relatively low rate of environmental variance (average within-RIL standard deviation equated to about 6% of mean rosette RGR) whilst roots and petals had higher levels of

environmental variance (17% for roots and 16% and 13% for petals in the Bay x Sha and L*er* x Col populations, respectively).

These observed differences in environmental variance between traits could be explained by differences in the extent of environmental differences in the three experimental areas. However, previous analysis showed that the glass house area used for petal area QTL analysis had a more constant environment across space than either of the areas used for root and rosette growth measurements – that is light intensity and temperature were seen to vary less across the space at a given point in time. Because of the natural day-light conditions and lack of stringent temperature controls, there will have been more fluctuations in environment in this area than in internal growth rooms. However, the complete set of RILs was grown at one time in the petal area analyses, such that all plants simultaneously experienced any general fluctuations in environment, whilst root and rosette analyses were carried out in blocks, which potentially introduced additional environmental fluctuations between RIL environments. This suggests that the environmental variance observed in petals was not due to an increase in environmental differences within the experiment.

The estimate of environmental variance (V_E) in these analyses includes all factors other than genetic variance – that is environmental factors such as nutrition, light quantity and quality, water and temperature, as well as maternal factors (e.g. seed-size), measurement errors and other stochastic ("normal") variation that cannot be explained by any of these factors directly. Any genetic-environment interactions are also included in this estimate. Assuming that the environmental factors were equally or better controlled in the petal growth experiments than root or rosette growth, that maternal factors were equivalent (as seed-size was not selected for in any of these analyses) and that measurement errors are a minimal source of variance, so can be ignored, then the increased V_E in petal area compared to rosette RGR must be due to either increased stochastic variation or increased genetic-environment interactions.

An increase in stochastic variation – the seemingly random, intangible variation between individuals – could occur due to a lack of stringency in control of the trait. In other words, if rosette RGR is tightly controlled by genetics and genetic-

environment interactions, stochastic variation would be low: this would reflect the importance of this trait in contributing to plant fitness in local conditions. Correspondingly, if petal growth, as a low contributor to fitness, were less stringently controlled, a high level of stochastic variation would occur, thereby enhancing the estimate of V_E in the experiment.

Alternatively, increased genetic-environment interactions might occur in petal growth relative to rosette growth, also causing an increase in the estimate of V_E. Genetic-environment interactions have been observed to affect floral morphology: analysis of the flowering of different phytochrome mutants under varying red:far-red ratios showed that *A. thaliana* flowers could exhibit phenotypic plasticity in response to changes in light quality (Weinig, C., 2002). As well as environmental differences during flowering phase inducing differences in petal area, differences in the local environment of individuals during the vegetative growth of the plant might contribute to floral morphology differences in a cumulative manner by genetic-environment interactions causing variation in the preparation of individuals for the flowering phase. The increase in V_E observed in petals compared to rosettes might, therefore, be explained by this simple increase in duration of the experiment, increasing the amount of environmental fluctuations experienced by individual plants over the course of the experiment.

Of these two hypotheses, the latter could be tested by analysis of genotypes in different controlled environments, probably requiring more stringent environmental controls than have yet been achieved in these QTL analyses. Although a reduction in stringency of control of petal growth may increase the stochastic variation in this trait, it seems unlikely that this difference alone would account for the large difference in V_E observed between rosette RGR and petal area. Also, as seedling root growth is a major contributor to early plant fitness, it is probable that the V_E observed in root length is due to a high level of genetic-environmental interactions. Taken with the observations of floral plasticity in response to their immediate light quality environment (Weinig, C., 2002) and the differences in QTL identified by previous studies of two RIL populations (Juenger, T. *et al.*, 2000; Juenger, T. *et al.*, 2005), these observations suggest that genetic-environmental interactions do affect

the growth of petals and may, therefore, be at least partly responsible for masking QTL effects in the above QTL analyses.

8 The use of STAIRS to locate QTL

8.1 Introduction

Recently, a new resource was developed for simplification of QTL analyses: Stepped Aligned Inbred Recombinant Strains (STAIRS; Koumproglou, R. *et al.*, 2002) allow systematic analysis of chromosome regions for the presence of QTL effects. The L*er* x Col set of STAIRS were created by first introgressing single L*er* chromosomes into a Columbia background, thereby generating chromosome substitution strains (CSS). Through backcrossing CSS to Columbia and genotyping at selected markers on each chromosome, lines were selected with single crossover events, such that lengths of Columbia chromosome were introgressed into the L*er* chromosome of the CSS. The result was a set of lines for each chromosome where each line carried a different length of L*er* genotype introgressed into the Columbia background (Koumproglou, R. *et al.*, 2002; see Figure 2.3).

STAIRS can be used to roughly map QTL using a small sample number. Firstly, comparisons of CSS with Columbia allow identification of which chromosomes exhibit a QTL effect. Each marker-delimited region is then analysed for presence of a QTL effect by pair-wise comparison of STAIRS for the chromosome of interest. When a QTL has been successfully mapped, the confidence interval can be reduced by creating fine-STAIRS within this region: two STAIRS that segregate for the QTL are crossed to produce new recombinants within the region of interest and genotyped at multiple markers across this region. The size of confidence interval, as in all QTL analysis, is dependent on the number and coverage of markers used.

One great advantage of STAIRS as a means of QTL analysis is that pairs of genotypes, rather than large RIL populations, are compared at any one time. This allows an increase in sample number whilst maintaining a small experimental area, so as to obtain a more accurate estimate of the mean of a trait and reduce environmental variation. Pairs of STAIRS segregating for a QTL could also be used in a similar manner to near isogenic lines, such as HIFs, to analyse QTL effect under a variety of conditions and environments, before exact position or identity of the

QTL is known. A limitation of STAIRS, however, is that each chromosome region is analysed for the presence of a QTL in only one genetic background, meaning that epistatic interactions cannot be detected.

Analysis of rosette leaf number at day 30 (RLN30), flowering time (FT) and plant height at day 30 (Ht30) illustrated the potential of STAIRS for identifying QTL (Koumproglou, R. *et al.*, 2002): a QTL was identified on chromosome 3, between 0 cM and 44 cM, the Columbia allele of which caused an increase in RLN30, a delay in FT, and a decrease in Ht30. The likelihood of this QTL was enhanced because a previous QTL analysis for flowering time had identified a QTL of similar effect within this region (Jansen, R. C. *et al.*, 1995). Also, a number of candidate genes for flowering time were identified between 0 cM and 20 cM on chromosome 3 (Koumproglou, R. *et al.*, 2002).

In this analysis, chromosome 2 was selected for screening for a QTL for petal size, rosette RGR and root length in the L*er* x Col STAIRS population because of the polymorphism in the *ERECTA* locus at 50.64 cM. A previous QTL analysis for petal length in the L*er* x Col RIL population indicated the presence of two QTL of moderate to large effect on this chromosome, one localising to the *erecta* mutation (explaining 30% of the total variance), the other being located at 10 cM relative to the L*er* x Col genetic map and explaining 10% of the total variance (Juenger, T. *et al.*, 2000). The *ERECTA* locus also affected leaf growth traits in previous QTL analyses (Juenger, T. *et al.*, 2005; Kearsey, M. J. *et al.*, 2003; Perez-Perez, J. M. *et al.*, 2002). *ERECTA,* therefore, acted as a test locus in these analyses, with detection of a large-effect QTL predicted in rosette and petal analyses. Simultaneously, novel QTL could be detected by pair-wise analysis of other regions of chromosome 2 with a fixed *ERECTA* background genotype.

The aims of the following analyses were therefore to verify the usefulness of STAIRS as a genetic resource for mapping QTL (particularly in view of the advantage of reduced environmental variation) and to identify novel QTL for growth traits in a L*er* x Col background. Chromosome 2 STAIRS were randomly assigned

letters A-P for identification during the analysis (see Materials and Methods 2.7.3 including Figure 2.3).

8.2 Petal size in Landsberg *erecta* x Columbia STAIRS for chromosome 2

8.2.1 QTL effects for petal size

L*er* x Col STAIRS for chromosome 2 were analysed for petal size QTL. Plants were grown under long-day conditions according to the method described in Materials and Methods 2.2.2 and petals from the third to the twelfth flower measured (see Materials and Methods 2.3). Sample numbers (total petals measured per STAIRS) varied from 10 for line J to 53 for line G with an average of 29 petals sampled per line. Overall mean petal length was 2.19 mm. Sections of the chromosome were analysed for presence of a QTL by comparing petal measurements from relevant pairs of lines by means of an unpaired t-test. This initial experiment identified three regions as carrying putative QTL effects for petal length (see Table 8.1). One of these regions spanned the *ERECTA* locus, for which an effect was also observed when all lines with a visible *erecta* phenotype were compared with all wild-type lines (unpaired t-test: $p < 0.01$; mean *ERECTA* petal length – mean *erecta* petal length = 0.40 mm). The likely positions of the other two QTL were between 1.75 cM and 9.60 cM and between 50.65 cM and 73.77 cM. Thus the downstream QTL could co-localise with that identified by Jeunger *et al* (2000) between 61.4 cM and 69.9 cM, whilst the upstream region potentially carries a novel petal length locus. A caveat of these results is that only one pair-wise comparison (M v H) was available to demonstrate the downstream QTL and all three significant results for the upstream QTL involved comparison of line L to another STAIRS. Had other segregating STAIRS for these regions been available, the significance of the test would have been enhanced if they had verified these effects.

Putative QTL	Pair-wise comparison	Segregating region (cM)	Petal length difference (Col - Ler; mm)	Unpaired t-test p-value for difference
1	L vs B	1.75 – 35.04	-0.18	0.014
1	L vs I	1.75 – 35.04	-0.30	0.000
1	L vs C	1.75 – 9.60	-0.28*	0.000
2	J vs E	9.60 – 50.65**	0.26	0.002
2	J vs F	9.60 – 50.65**	0.25	0.003
2	J vs K	35.04 – 50.65**	0.25	0.003
2	H vs K	35.04 – 63.02**	0.34	0.000
2	G vs N	35.04 – 73.77**	0.41	0.000
2	D vs N	35.04 – 73.77**	0.35	0.000
3	M vs H	50.65 – 73.77	-0.27	0.000

Table 8.1. Significant pair-wise comparisons of *Ler* x Col STAIRS.
The STAIRS carrying the Columbia allele of the segregating region is listed first in the pair-wise comparison column. Additive effects are relative to the Columbia allele.
*This effect is due to a difference in breakpoint position between two non-segregating markers, so its direction is indefinite.
**These segregating regions contain the *ERECTA* locus; pairs of STAIRS differed for the *ERECTA* phenotype.

The two putative QTL (excluding the *ERECTA*-linked effect) were further investigated by twice re-analysing the relevant strains: C and L for the upstream QTL (1.75 cM- 9.60 cM); M and H for the downstream QTL (50.65 cM – 73.77 cM). Plants were grown under the same conditions as before and petals were collected from the third to twelfth flowers for measurement. Both repetitions of the experiment, however, failed to identify a significant effect in either of the putative QTL regions according to unpaired t-tests between pairs of STAIRS (see Table 8.2). Parental plants were also analysed as controls, although they could not be directly compared with STAIRS lines as they were not the specific strains from which the STAIRS were produced. Columbia and Landsberg *erecta* lines were found to differ significantly for petal length on both occasions (see Table 8.2). This suggested that the experimental conditions were sufficient to detect genetic effects on petal size and

therefore that the indication of significant QTL effects in the original experiment were false-positive results. These false-positives could have arisen due to environmental bias between genotypes, the probability of which is increased by the lack of alternative pair-wise comparisons to demonstrate the QTL effects.

	Repetition 1		Repetition 2	
Pair-wise comparison	Difference in means (mm)	p-value for unpaired t-test	Difference in means (mm)	p-value for unpaired t-test
L v C	0.05	0.163	0.02	0.801
M v H	0.00	0.848	0.03	0.666
Col v Ler	0.25	0.000	0.22	0.001

Table 8.2. Results of pair-wise comparisons to test for significance of two putative QTL-containing regions for petal length in L*er* x Col STAIRS. Significant differences of similar magnitudes between parents act as a control for the experiments. No significant differences in petal length were observed for the pairs of STAIRS representing the putative QTL differences.

8.2.2 Petal length plasticity

Analysis of petal length according to flower number showed that there was an overall significant floral-stage effect on petal size. Flowers were grouped into three categories: (a) flowers 3 – 6; (b) flowers 7 – 9; (c) flowers 10 – 12. Petal length varied significantly between categories, showing an overall reduction in length with increasing flower number (see Table 8.3, Figure 8.1). This difference (detected by unpaired t-tests) was dependent on genotype: line L showed a significant difference between petal lengths of groups (a) and (b) ($p < 0.01$); the difference between petal lengths of groups (b) and (c) was significant in line M only ($p < 0.05$); and the difference between groups (a) and (c) was found to be significant in line H ($p < 0.05$). Thus, plasticity in petal size appears to be both genotype- and floral-stage-dependent. Therefore, a bias in positions of flowers collected from each genotype during the original experiment could have resulted in significant differences in petal size and detection of false positive QTL. Although all flowers collected were between the third and twelfth flower on the main inflorescence, specific flower numbers were not recorded during the original petal collection. A bias could therefore have occurred, particularly if there were a difference in flowering time

between lines, such that inflorescences were at different stages of development at the time of flower collection.

	3-6 v 7-9	7-9 v 10-12	3-6 v 10-12
Cumulative values	0.000	0.000	0.000
C	0.794	0.582	0.470
L	0.003	*	*
M	0.897	0.031	0.130
H	0.101	0.443	0.010

Table 8.3. Petal length variation by flower number.
P-values for unpaired t-tests between pairs of flower number categories (3-6, 7-9 and 10-12) showing cumulative values (petal lengths for all four STAIRS) and separate tests for each STAIRS.

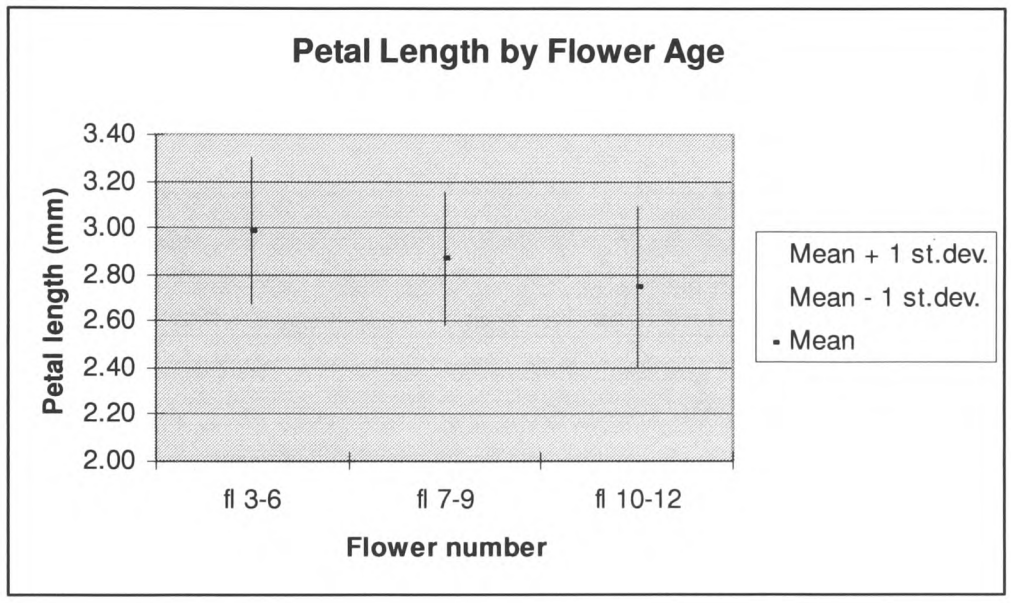


Figure 8.1. Petal length distribution.
Petal lengths by flower number, showing mean and spread of one standard deviation either side for cumulative petal lengths over STAIRS L, C, M and H. A general decline in petal length with age can be seen. Over 300 petals per flower stage (see appendix).

8.2.3 Flowering time

A difference in flowering time was observed between lines L, C, H and M and between parental lines. Columbia plants showed delayed flowering compared to Landsberg *erecta* plants (data not shown). Also, STAIRS L and C showed delayed flowering in comparison to STAIRS M and H and line L was slower flowering than line C (see Figure 8.2). Therefore, differences in flowering time could have

contributed to differences in petal lengths during the original data collection via differences in the flower numbers collected for measurements. Also, thrips (insects, which can cause flower damage and reduce fertility by feeding on the pollen) were observed on some plants during the original experiment, which could have interfered with petal measurements if they affected flower development non-uniformly.

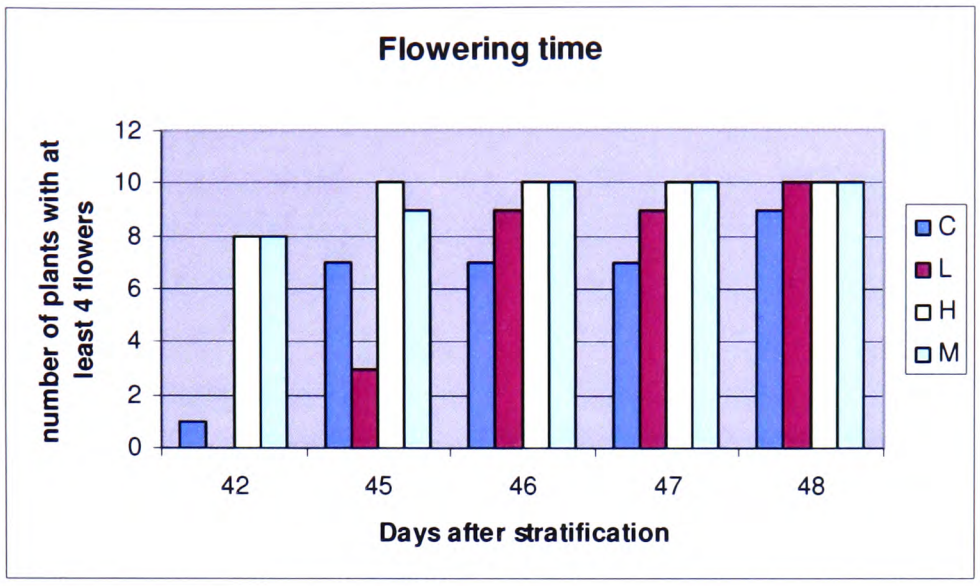


Figure 8.2. Flowering time in STAIRS C, L, H and M.
Illustrated by the number of plants having at least four flowers for 42 – 48 days after stratification. On day 42, eight of ten plants from lines H and M have reached the fourth flower, and by day 46 all plants of these lines have produced four flowers Lines C and L show a delay in flowering, only reaching four flowers in nine or ten of ten plants by day 48, with flowering in L also delayed in comparison to line C.

8.3 Rosette RGR in the L*er* x Col STAIRS for chromosome 2

8.3.1 QTL effects for rosette RGR

Rosette RGR measurements for the L*er* x Col STAIRS for chromosome two were carried out on short-day grown plants. RGRs over the period of growth from day 14 until day 24 after stratification were calculated for individual plants and pairs of STAIRS were compared using an unpaired t-test. The overall mean RGR during this experiment was 0.20 ±0.04. Parental lines showed a significant difference in RGR (mean Col RGR – mean L*er* RGR = 0.026; p-value = 0.000). The *ERECTA* locus showed a significant effect on RGR in only one of three relevant pair-wise comparisons. The difference between all lines with *erecta* phenotype relative to wild-type phenotype was small (mean *ERECTA* RGR – mean *erecta* RGR = 0.007)

but significant according to an unpaired t-test ($p = 0.0054$). An effect on RGR was detected in a further three regions by one or two pair-wise comparisons (see Table 8.4), however, these effects only appeared at a 5% probability level, not at a 1% level, and were not confirmed in comparisons between other lines, differing for the same region. The overall likelihood of these QTL effects was therefore low.

Putative QTL	Pair-wise comparison	Segregating region (cM)	RGR difference (Col - Ler)	Unpaired t-test p-value for difference
1	Col v L	0 – 9.60	0.019	0.016
2	H v K*	35.04 – 63.02	0.015	0.014
3	H v J	50.64 – 63.02	0.015	0.025
4	Col v M	63.02 – end	0.014	0.028
4	N v P	63.02 – 73.77	0.014	0.040

Table 8.4. Significant pair-wise comparisons of L*er* x Col STAIRS for rosette RGR.
Putative QTL 1-3 are only evident in one pair-wise comparison, whilst the effect of putative QTL 4 was significant twice.
*This analysis compares an *ERECTA* line with an *erecta* line.

8.3.2 Heritability during the experiment

To estimate heritability in this experiment, standard deviation of RGR was compared for within STAIRS and within trays. The standard deviation within trays is due to both genetic and environmental factors (each tray contained one plant of each genotype); whilst the standard deviation within STAIRS is due to environmental factors alone (genetic variation is removed as all samples within a strain have identical genotypes). Hence, if there were high genetic heritability of RGR, an increased standard deviation within trays compared to within STAIRS would be expected. An unpaired t-test for standard deviations by STAIRS compared to by tray gave a p-value of 0.78, indicating that there was no significant difference between the degree of variation within and between genotypes – i.e. that genetic variance did not significantly affect overall phenotypic variance. Broad-sense heritability $\left(\frac{V_G}{V_P}\right)$

was estimated at a very low 2.6%, which confirms this lack of genetic influence on the trait in this experiment.

8.4 Root Lengths in the Ler x Col STAIRS for chromosome 2

L*er* x Col STAIRS were also used to search for root length QTL on *A. thaliana* chromosome 2. Roots were grown as described in Materials and Methods Section 2.2 and growth was measured between three and twelve days after stratification. No significant QTL were detected in any of the regions of chromosome 2 by pair-wise comparisons of STAIRS.

8.5 Fine mapping breakpoints in one STAIRS region

Two STAIRS were selected for genotyping to fine-map the endpoints of the L*er* introgression into Columbia chromosome 2. Lines L and C had been previously genotyped at seven markers along chromosome 2, indicating that they both carried a L*er* introgression from the upstream end of the chromosome until some unspecified position between markers RGA (1.75 cM) and nga1145 (9.60 cM) (Koumproglou, R. *et al.*, 2002). New simple sequence length polymorphic markers were chosen between RGA and nga1145. DNA was extracted from STAIRS L and C and Columbia and Landsberg *erecta* plants; extracts were then genotyped at the new markers by PCR and agarose gel electrophoresis as described in Materials and Methods 2.6 (see Figure 8.3). Genotypes of L and C lines at the polymorphic markers were determined by comparing band sizes produced by PCR to Col and L*er* controls. The markers selected, their physical positions on chromosome 2 and the determined genotype for STAIRS L and C are shown in Table 8.5.

Genotyping these markers narrowed the confidence interval for the end of the L*er* introgression in line C to between 400 kb and 552.4 kb, but showed that three breakpoints had occurred in line L, such that the first Ler / Columbia breakpoint occurred between 255 kb and 283.5 kb, but that a further island of L*er* DNA had been incorporated downstream, around the marker T8O11b at 336.5 kb. Thus, a QTL effect between this pair of STAIRS could occur in either of two regions for

which segregation is observed - between 255 kb and 336.5 kb, or between 336.5 kb and 552.4 kb. Further genotyping around marker T8O11b (at 336.5 kb) would allow estimation of the size of this downstream L*er* island, allowing this region to be removed from the confidence interval of the QTL effect.

Marker	(RGA)	T8O11a	T8O11b	T23K23a	T23K23b	F504	T16F16	(Nga1145)
Map position (kb)	(255)	283.5	336.5	388	400	552.4	596	(684)
Genotype in L	(L*er*)	Col	Ler	Col	Col	Col	Col	(Col)
Genotype in C	(L*er*)	Ler	Ler	Ler	Ler	Col	Col	(Col)

Table 8.5. Physical map positions and genotypes of six polymorphic markers in STAIRS L and C.
(RGA and nga1145 had been previously genotyped L*er* and Col respectively in both lines.)

Figure 8.3. Genotyping of STAIRS.
Band sizes of PCR results run on 3% Agarose gels for markers corresponding to Table 8.5.

8.6 Discussion

L*er* x Col STAIRS were used to detect QTL for petal length, rosette RGR and root length on chromosome 2. This resource was thought to be advantageous because the

reduction in experimental area required (compared to QTL analysis by RIL populations) should lead to a reduction in environmental variance and hence an increase in the power of the experiment in detecting genetic effects. Also, STAIRS analysis allows simple interpretation of data by pair-wise comparisons. The detection rate of QTL might be decreased, however, by the limitation of genetic background, such that epistatic interactions of QTL cannot be detected.

Three QTL effects on petal length were detected in an original experiment. One of these effects co-localised with the *ERECTA* locus, and was also observed in a comparison between all *ERECTA* and *erecta* phenotyped lines. The *erecta* mutation correlated with an average decrease in petal length of 0.40 mm relative to a mean petal length of 2.19 mm. The direction of this effect equates to that observed previously (Juenger, T. *et al.*, 2000), suggesting that this is a real effect. The two other QTL detected in this analysis, however, could not be confirmed as there was no consistency of effect observed in repetitions of the experiment. It is most likely that these two effects were false-positive results of the first experiment, caused by environmental variation, perhaps due to the thrips infection observed in some plants, or by floral stage-related plasticity along with flowering-time variation. Differences in petal size correlated with flower number, even within the first twelve flowers produced by the plant, an effect that also varied according to genotype. Flowering-time variation was also observed across STAIRS. This suggested that lack of stringency in the first experiment could have caused a bias in flower number collected in some genotypes compared to others, resulting in a flower-number related effect on petal length.

Heritability for rosette RGR was very low (2.6%) in the STAIRS analysis. Although a small *ERECTA*-linked effect was observed (additive effect = 0.007; $p = 0.0054$), this was not apparent in two out of three relevant pair-wise comparisons of STAIRS. Likewise, probabilities of other putative QTL were greatly reduced by the lack of consistency between pair-wise comparisons. Similarly, no QTL effects were observed for root growth in this set of STAIRS. This lack of QTL detection could be caused by many environmental differences acting over the experiment so as to mask any genetic effects. This would correlate with the low heritability, but environmental

differences are more likely to have been reduced in this experiment, relative to QTL analysis by RILs, because a smaller experimental area was used. Also, it may be that no QTL are present for these traits in chromosome 2 of the L*er* / Col cross, or that existing QTL have very small effects, such that they have not been identified in these analyses. Conversely, there could be multiple QTL of antagonistic effects present in chromosome 2 and the lack of detection of QTL in the STAIRS could be due to an absence of recombination events between QTL, so that regions contain two or more QTL of antagonistic effects. Thus QTL effects would be cancelled out within STAIRS regions and no phenotypic effect would be observed. In order to avoid such clustering of antagonistic effects, further recombinations and finer genotyping would be required to break up the large STAIRS regions.

Such close-linkage of QTL was seen in a fine-mapping analysis of growth rate in a 210 kb interval in a L*er* / Col cross (Kroymann, J. & Mitchell-Olds, T., 2005). There was originally no evidence for growth rate QTL within this interval; however, on fine-mapping using NILs, two QTL were discovered. Because of the tight linkage of these QTL, and because they displayed strong epistasis (the direction of effect of the upstream QTL changed with genetic background, whilst the downstream QTL could only be detected in a specific genetic background) a normal QTL analysis would not have identified their effects. The identification of these two QTL within a small, effectively randomly selected, region of the genome, suggests that quantitative traits could be controlled by many small effect QTL with complex linkage and epistatic effects, thus contributing to the difficulties of QTL mapping (Kroymann, J. & Mitchell-Olds, T., 2005).

The usefulness of STAIRS as a QTL mapping resource depends in part on the reliability of predictions of chromosome status between the genotyped markers. It is assumed that only single crossovers occur in each chromosome, such that simple, single introgressions of L*er* DNA into the Columbia chromosome can be identified (Koumproglou, R. *et al.*, 2002). However, if the distances between markers are too large, 'invisible' recombination events may occur, resulting in a mixing of genotypes that is not recognised during analysis of the STAIRS. This in turn would disrupt pair-wise comparisons, reducing the power of the analyses in detecting QTL.

Evidence to support this hypothesis comes from genotyping within a single STAIRS region. Two STAIRS lines were selected, which had been previously genotyped to suggest breakpoints of the L*er* introgression between markers RGA and nga1145 at 255 kb and 684 kb on chromosome 2, respectively. By genotyping these lines at new markers within this region, the breakpoint was identified in one line to lie between 400 kb and 552 kb. However, genotyping the other line showed that recombination had occurred three times within this region, leading to an island of L*er* DNA downstream of the first L*er* / Col breakpoint. If this additional mixing of parental genotypes within STAIRS regions is a common event, the pair-wise genotype comparisons carried out during analyses will have inconsistent results due to unresolved mixing of genotypes.

Although *A. thaliana* was previously thought to have a low genome-wide recombination rate (Copenhaver, G. P. *et al.*, 1998), a recent in-depth analysis of crossover events on chromosome 4 has identified a wide variation in recombination rates across the chromosome, including interspersed 'cold' and 'hot' regions, where a lower or higher rate of recombination was observed compared to the expected chromosome-wide average (Drouaud, J. *et al.*, 2006). This supports the hypothesis that unidentified mixing of genotypes might have occurred during production of STAIRS. More, closely spaced markers would be needed to increase confidence of the chromosome identity within STAIRS regions.

In conclusion, it appears that STAIRS have not been as useful a resource for mapping the quantitative traits of petal size, rosette RGR and root growth as was predicted. Rather than a decrease, an increase in environmental variance has been observed during the growth experiments, with a corresponding decrease in heritability. Therefore, the expectation of reducing environmental variance, increasing heritability and hence increasing power of analyses by reducing the area of the experiment has not been met. Fine-mapping within a STAIRS region suggests that STAIRS might carry more complex genotypes than was previously predicted, with additional, unmarked, recombination events occurring within regions, perhaps where 'hotspots' of recombination exist. This would result in additional mixing of

genotypes, reducing the power of analyses and therefore limiting the usefulness of this QTL-mapping resource.

9 Conclusions

This quantitative analysis of *A. thaliana* growth has studied three organs of the plant – roots, leaves and petals – and has employed QTL mapping techniques using two RIL populations, HIFs and STAIRS. Two putative QTL for root growth rate and three for petal size were identified at the 1% significance level during QTL analyses. Various limitations prevented confirmation and fine-mapping of these putative QTL: particularly the small effects of QTL, the high plasticity of *A. thaliana* growth rates and the inability to precisely control the environment over the experimental area. It seems likely that multiple, small-effect QTL are responsible for control of growth rates and that many of these will be environment-specific or involve genotype-environment interactions. This would enable plants to become 'fine-tuned' to a wide variety of environmental cues, such as changes in light quantity or quality, water and nutrient availability and temperature (Hoffmann, M. H. *et al.*, 2005; Malamy, J. E., 2005; Smith, H. & Whitelam, G. C., 1997). Such plasticity is an important characteristic for an opportunistic annual plant, such as *A. thaliana*.

9.1 Limitations of the Study

A number of limitations reduced the efficacy of this study and can cause difficulties in QTL analyses in general. These limiting factors are: the measuring techniques; the environment; time and effort; and qualities of the populations under study.

9.1.1 Measurement Techniques

The measurement techniques developed for the study of root and rosette growth rate were non-destructive. These are of greater advantage than destructive methods of analysis because they allow for an increase in the number of time points used during the experiment – thereby increasing the accuracy of measurements – without the need for a large increase in the number of plants, as would be required with destructive analyses.

Root growth rate analysis was straightforward, with a high degree of accuracy achievable as root tip positions were marked on a daily basis and measurements

carried out by computer analysis of the final image. Errors due to differences in germination timing were avoided by measurement of all roots from the third day of growth, rather than from emergence of the radical.

Rosette RGR measurements were developed to avoid the effects of leaf-overlap, which significantly affected measurements after four weeks of growth, in the conditions under study. The accuracy of measurements was limited mainly by the consistency of the user during the process of image selection. In these experiments, measurements were very consistent between repetitions; however greater discrepancies might arise if multiple users were involved.

Petal growth rate could not be measured, as these organs only become accessible when they reach maturity and reflex from the flower bud. Thus, petals were removed from flowers at the fully opened stage and final petal area was measured. A major limitation involved in petal measurement was the variation in the exact position of the break when petals were removed from flowers. Also, again, the accuracy of selecting the petal area in the photographic image was a limiting factor in these measurements.

9.1.2 Time and Effort

There was considerable time and manual effort involved in extracting data from photographs, which acted to limit the number of time-points practical and therefore the accuracy of growth rate calculations. In order for rosette and petal measurements to become more efficient, increased computer programming would be required to automate many of the tasks. This would also reduce the judgement error of measurements by eye.

The number of samples required for any QTL analysis means that such experiments are bound to be time, space and effort consuming. Furthermore, an in-depth analysis of a trait would require the experiment to be repeated in several environments with several populations and large follow-on experiments would be needed to confirm and isolate any QTL. Although the non-destructive measurement techniques and the use

of STAIRS and HIFs reduced the sample numbers required, these QTL analyses remained highly labour-intensive.

9.1.3 Populations

The lack of QTL identified during these experiments could be due to a lack of QTL present in the populations under study. The Bay-0 x Shahdara RIL population was chosen because a good number of well-genotyped lines were available, from which HIFs could be easily identified for follow-up analyses. Variation was observed for growth rate in all three organs and the population had undergone previous QTL analyses successfully, including an analysis of root growth, which identified three QTL for primary root length (Loudet, O. *et al.*, 2005). The second RIL population, Landsberg *erecta* x Columbia, was a long-established RIL population, which had been used in many previous QTL studies. This population had the advantage of both parental genotypes being publicly available, so that new markers could be identified for fine mapping of QTL confidence intervals. The presence of the *erecta* mutation was initially thought to be disadvantageous, because of the potential of this large-effect locus to mask small-effect QTL; however it became a useful control during petal-size experiments as it was predicted to have a large effect on this trait. As discussed earlier (see Introduction 1.1.4) most 'successful' QTL analyses involve the cloning of major effect mutations, such as the *erecta* mutation, however the majority of natural variation for quantitative traits is likely to involve multiple small-effect QTL, allowing stabilising selection during adaptation, as opposed to the rare incorporations of large-effect mutations.

Multiple populations are required for an in-depth analysis of any quantitative trait, as QTL will only become apparent when they vary in the parental genotypes. A lack of detection of QTL might illustrate that the two parental strains are genotypically similar for the trait of interest. However, as there were clear phenotypic differences between parents in both RIL populations, when grown in a common environment, it is unlikely in this case that there were no genotypic differences present. Rather, it may be that all QTL present were of particularly small effects, making detection difficult.

9.1.4 Resources

All three resources used – RILs, HIFs and STAIRS – are limited by the accuracy of genotype prediction, which depends on the distance between markers and the frequency of recombination events. Although double cross-overs between markers will be rare (providing markers are reasonably close together), they would cause a false prediction of QTL genotypes. This effect would be minimal in a RIL population, as it is likely to occur in only a small number of RILs, thereby only introducing a small disruption to the total number of genotypes predicted. But it would be magnified in STAIRS and HIFs, where fewer individuals are genotyped and, therefore, the importance of predicting each genotype correctly is greatly increased.

Genotyping one STAIRS region during this study suggested that such events might be more common than previously predicted and are possibly responsible for some of the difficulties met in predicting and verifying QTL in these experiments.

This agrees with two recently-published papers in which high-density mapping of the Bay x Sha and L*er* x Col RIL populations is described. The Bay x Sha population was fine mapped using 188 gene expression markers (GEMs) and 599 single feature polymorphisms (SFPs) detected by microarrays (West, M. A. L. *et al.*, 2006). This produced very high marker coverage of the genome compared to the original 38 microsatellite markers, which detected 836 crossover events during production of the 148 RILs analysed. In contrast, the SFP map identified 1533 crossovers. Therefore, using the 38 microsatellite markers, the unidentified recombination events will result in some QTL genotypes being incorrectly predicted during QTL analyses. Similarly, 100 L*er* x Col RILs were genotyped using a high-density SFP marker map which carried almost 16,000 markers (Singer, T. *et al.*, 2006), compared to the 88 AFLPs used in this study. Unexpectedly, this high-density mapping led to a large decrease in predicted crossover events when compared to a map derived from 242 publicly available marker data. One suggested explanation for this is that there are genotyping errors in the public data, which might also explain the low significance of

QTL identified in this study. Also, there may be recombination events detected in these maps that are not evident in the map generated using 88 AFLPs.

A further advantage of high-density mapping is that QTL can be mapped to within smaller confidence intervals. The high-density L*er* x Col map had an average interval size of 0.62 cM, with many intervals containing only one gene (Singer, T. *et al.*, 2006) whilst the high-density SFP map for the Bay x Sha RIL population had, on average, one marker every 0.64 cM (West, M. A. L. *et al.*, 2006). The use of high-density maps in these QTL analyses would potentially increase the power of the tests by identifying further recombination sites and reduce the confidence intervals of QTL, allowing candidate gene identification to be more readily carried out.

9.1.5 Environmental fluctuations

The major difficulty in this study arose in trying to maintain a constant environment during QTL analyses. Several available controlled growing areas were tested, but environmental fluctuations were a factor in all areas. A trade-off in sample numbers was required – an increase in the number of repetitions would increase the statistical significance of measurements for each genotype, but would also require a larger experimental area, thereby potentially increasing the fluctuations of the environment across the experiment. Light intensity, temperature and humidity all showed variation across the controlled growth areas and have the potential to alter growth rates. These environmental fluctuations were likely largely responsible for the low QTL detection and the lack of repeatability of the experiments, because of the high degree of plasticity in the traits. Therefore, QTL analyses of such highly plastic traits require very precisely controlled environments. Analysis under multiple controlled environments would also allow elucidation of environment-dependent effects.

9.1.6 Seed history

The origin and history of the seeds used in these QTL analyses may also have affected the results. Bulked F8 Bay x Sha RIL seeds were obtained from Olivier Loudet, being decanted and dispatched from the INRA in Versailles on request in

December 2003. Col x L*er* RIL seeds were donated by Catherine Kidner. These seeds were older and had travelled from the US after being bulked in 2000. That each set of RIL seeds was included the progenitors of multiple parents decreases the likelihood of maternal effects greatly influencing seed traits, although traits such as seed germination can be affected by the environmental conditions experienced by the maternal plant (van der Schaar, W. *et al.*, 1997). This adds a further consideration to QTL studies: traits should be studied not only within different environments, but also in seeds that have been harvested from different maternal environments to include possible maternal environment-QTL interactions.

During these analyses, Bay x Sha RILs all had good germination rates, but L*er* x Col RILs were more variable, suggesting that these seeds had suffered during storage and transportation, which may have added to environmental variance during these experiments.

9.1.7 Genotyping errors

A further explanation for the low significance of QTL peaks in these analyses is the possibility of genotyping errors having been incorporated into the RIL data. Such errors could have arisen during collection or dispensing of seeds or due to the mixing of data in the genotype databases. If a portion of RILs were incorrectly genotyped during analysis there would be a breakdown of the expected marker-trait association, leading to a reduction in significance of QTL peaks. The utmost care has been taken to ensure no confusion of genotypes during these experiments and the positions that were genotyped in the Bay x Sha RILs produced results consistent with the given data. However, to guarantee correct seed-genotype correlation, all RIL markers would require genotyping on receipt of seeds. This would be advisable before the commencement of any further experiments.

9.2 Summary of Results

Due to the limitations described above, the putative QTL identified in this study could not be verified or fine mapped. However, this preliminary study has identified

several small-effect QTL for the traits of root growth rate, leaf number and petal size (see Figure 9.1).

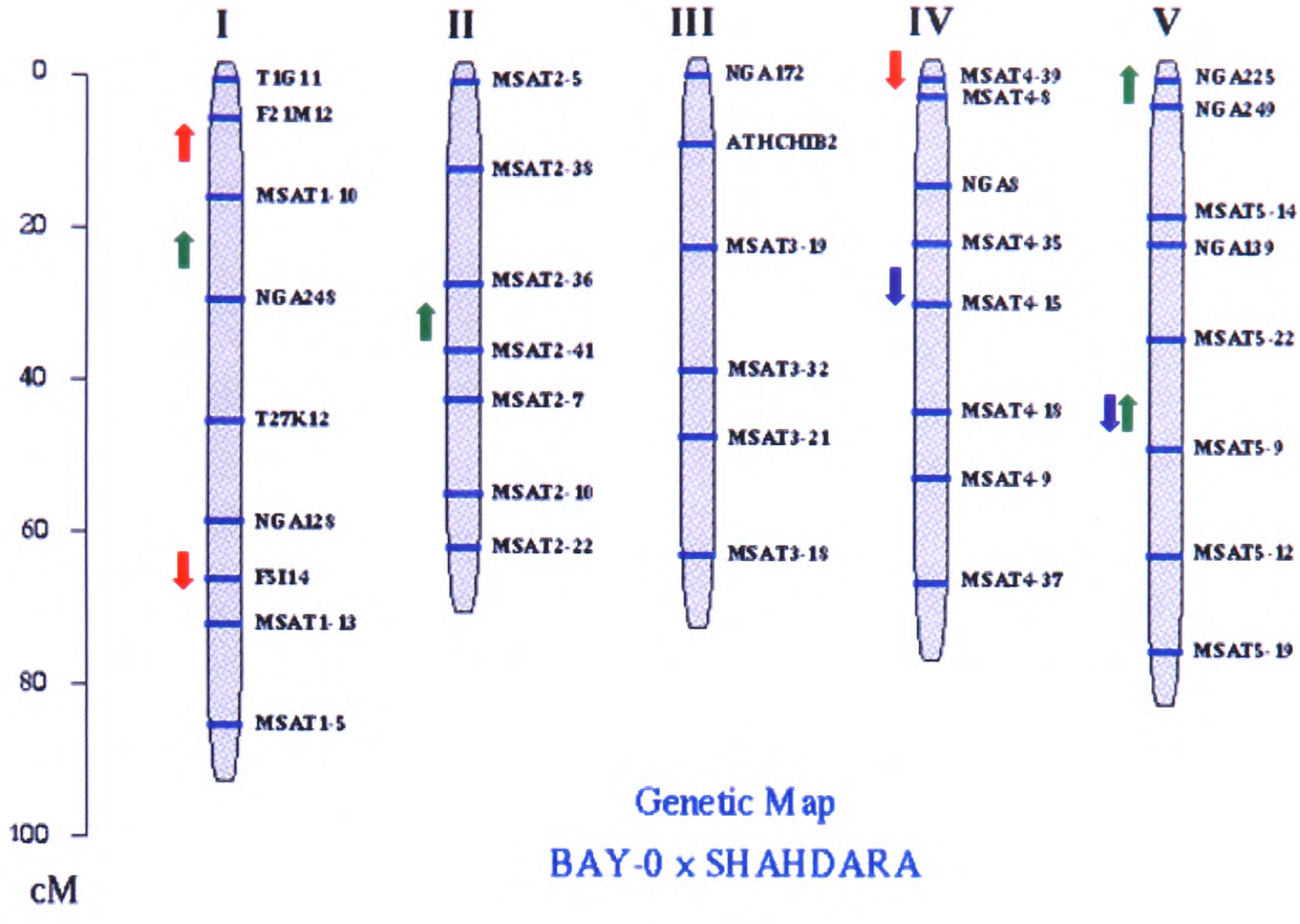


Figure 9.1. Putative QTL located in this study.
Pictorial summary of putative QTL ($p<0.05$) for (red) petal area, (green) leaf number at 32 days and (blue) primary root length from day 3 to day 10. Upward pointing arrows indicate an increase in the trait value by the Bay-0 allele; downward pointing arrows indicate that the Shahdara allele increases the trait value. (Picture adapted from Loudet, O. *et al.*, 2005).

The leaf number QTL on chromosome 5 at 45 cM and an additional putative leaf number QTL on chromosome 4 at 65 cM are supported by the co-localisation of putative rosette RGR QTL in these regions (although RGR QTL were all below the 5% significance threshold). For this study to be taken forward, highly controlled environments would be necessary to allow the precise repetition of experimental conditions for verification of QTL and fine-mapping.

The small effects of these QTL and their seeming environmental dependence suggest that they will not be useful candidates for the practical purposes of crop

improvement. However, they may be used to further the understanding of growth rate control in *A. thaliana*, which could then lead to the identification of genes which may be advantageously manipulated in crop genomes.

9.2.1 Candidate genes for putative QTL

A selection of candidate growth rate-related genes, as mentioned in Introduction 1.5, were tested for co-localisation with putative QTL from this study by plotting the genes of interest on to the TAIR sequence viewer (http://www.arabidopsis.org/servlets/sv) along with the bacterial artificial chromosomes (BACs) containing markers either side of each putative QTL. Most of the QTL did not co-localise with a candidate gene, but the position of the petal length QTL at 10 cM on chromosome one corresponded to the location of a gene encoding a putative cell division protein kinase, *CDKD1;3*. This gene is located on BAC T10F20, which is roughly halfway between the BACs containing markers F21M12 and MSAT1.10.

Further analysis of candidate genes would require the generation of confidence intervals for QTL peaks and identification of potential growth rate-regulating genes within such intervals. The putative QTL identified in this study were not found to co-localise with previously identified QTL in the Bay x Sha population.

9.3 QTL Analysis for Crop Improvement

At least theoretically, the identification of QTL allows for 'intelligent breeding' by marker-assisted selection, to improve qualities such as growth rate, yield and pest-resistance in crops (thereby potentially alleviating some of the need for genetic modification). However, over the course of these experiments several limitations have become apparent, which question the usefulness of QTL analysis in crop improvement. Firstly, QTL analysis remains a highly labour-intensive method of analysis, despite improvements in efficiency by use of resources such as RILs, HIFs and STAIRS, and the availability of the *A. thaliana* genome sequence. Secondly, QTL analysis on a model organism is only the beginning of a long journey to fine-map and identify the QTL, although a recent assessment suggests that much of the fine-mapping might be by-passed, because the positioning of the QTL by the primary

mapping is much more accurate than was previously considered (Price, A. H., 2006). The gene identified in the model organism must then be followed through to find the corresponding gene in crop plants by homology. This assumes that a copy of the gene exists in the crop plant with similar form and function. For intelligent breeding of the crop plant to utilise advantageous alleles of this gene, there would also need to be natural variation in the corresponding gene in the crop plant.

It has been suggested by these experiments that multiple small-effect QTL are responsible for the natural variation in growth rate observed in *A. thaliana*. In order to enhance the trait significantly, the incorporation of advantageous alleles at multiple loci would be required – a factor that further increases the difficulties of intelligent breeding. Furthermore, these QTL have proved highly evasive with environmental changes, suggesting a high degree of plasticity and genotype-environment interactions. Whilst providing a very uniform environment may allow the identification of loci involved in growth rate control, such loci may be of little benefit when exposed to natural environmental fluctuations in the field.

As an alternative to using model organisms, QTL analysis may be carried out directly in the crop. This is likely to increase the time, area and expense of the initial QTL analysis, however it allows for the utilisation of advantageous alleles present in wild relatives of domesticated crop species. For example, three loci were identified in the wild tomato species, *Solanum pennellii*, which, when introgressed together into the genetic background of *S. lycopersicum*, produced increased yield in this tomato crop species (Gur, A. & Zamir, D., 2004). This suggests that, during the domestication of tomato, beneficial alleles have remained hidden in the wild species; this is likely also the case in many other crops as QTL are often masked by epistatic interactions and so would not have been selected for during breeding programs. A major advantage of marker-assisted selection is that it allows 'pyramiding' of genes – that is the simultaneous selection of multiple QTL that together have a beneficial effect of the trait, but would not be detected separately by phenotypic selection (Collard, B. C. Y. *et al.*, 2005). The use of real crop species for the initial QTL analysis allows introgression of QTL regions by marker-assisted selection, without

the need for identification of the gene and locus variation responsible for the effect, thus accelerating the benefits of crop breeding by natural variation.

It seems sensible that, wherever possible, the elucidation of such alleles should take place in the crop of interest in its common environment. This would narrow QTL analysis to the finding of alleles that provide real benefits in the production of the crop. An exception to this might be in the improvement of stress-responses in crops, where the environment might be manipulated to produce a selective pressure on the plant, so as to identify alleles which protect against specific environmental stresses. For example, alleles that maintain or increase yield in response to high density growth would be useful for the increase of crop production required as world population increases; heat and drought-tolerant QTL would be highly beneficial with the prospect of global warming; and the introduction of pest-resistance QTL into crops could enhance yield and reduce pesticide usage.

9.4 Summary

It remains that QTL analysis is a useful approach to the detection of new genes and, at least when carried out in crops, opens possibilities of crop improvement.

However, analysis of growth rate has proven to be labour intensive and resource-demanding, the QTL being difficult to map because of their small effects and the high plasticity of the trait. If the aim is crop improvement, a more efficient approach would be to look for beneficial large-effect QTL in wild relatives of crop species, testing these under the natural environments to which they are most likely to be exposed. But for continuation of this analysis for furtherance of the understanding of growth rate control in *A. thaliana*, high-precision growth areas would be necessary. The putative QTL localised here could then be confirmed and assessed using new HIFs and the underlying genes identified by further genotyping, fine-mapping and testing of candidate genes.

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